

ABSTRACT

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THE ROLE OF MYXOVIRUS (INFLUENZA VIRUS) RESISTANCE A IN PROSTATE CANCER

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Myxovirus (influenza virus) resistance A (MxA) is an interferon regulated protein responsible for a specific antiviral state against viral infection. Our lab has previously shown that MxA is up-regulated by androgens in the normal prostate epithelial cells; however, there is no known role for MxA in cancer. Meta-analysis of different expression databases (e.g. NCBI GEO and Oncomine) suggested a strong inverse association between MxA expression and prostate cancer. To confirm these studies, we performed immunohistochemistry on normal prostate and prostate cancer tissues. Our results revealed that MxA expression was indeed decreased in cancerous as compared to normal prostate, indicating that MxA could be transcriptionally down-regulated in cancer. Previous studies indicated that MxA down-regulation could be due to a specific polymorphism in the proximal MxA promoter at position -88. This single nucleotide polymorphism G>T (rs2071430) is involved in modifying the gene expression and interestingly, it harbors an interferon-stimulated response element (ISRE) that is required

for expression in response to interferons. The “T” allele restores whereas the “G” allele attenuates ISRE binding, resulting in increased or decreased MxA expression, respectively. Based on these observations we hypothesized that decreased expression of MxA in prostate cancer could be due to the rs2071430 polymorphism. We investigated this polymorphism in genomic DNA from equal number of disease free and prostate cancer samples. The results provide evidence that the GG genotype (low promoter activity) is higher in PCa (72%) as compared to normal (58.6%). The TT genotype (high activity) was higher in normal (5.7%) compared to PCa (2.4%) $p < 0.05$. No TT genotype was observed in Caucasian normal samples. Our results, for the first time demonstrate that MxA is down-regulated in prostate cancer possibly at the transcriptional level due to ISRE polymorphism in the promoter region.

Recent studies have indicated that the antiviral protein MxA may have alternative functions including modulating apoptosis and inhibiting metastasis. The second aim of this study was to investigate the functional significance of MxA in prostate cancer. Results showed that MxA is regulated through the classical IFN pathway as well as by androgens and serum factors. DU145 cells lacking MxA showed a significant increase in proliferation, migration and a decrease in apoptosis as compared with the vector control, which suggests that MxA may play an important role as a tumor suppressor in prostate cancer. MxA may induce apoptosis in prostate cancer cells and sensitize them to chemotherapeutic drugs thus making MxA a promising target for gene therapy on prostate cancer.

THE ROLE OF MYXOVIRUS (INFLUZENA VIRUS) RESISTANCE A
IN PROSTATE CANCER

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LIST OF ABBREVIATIONS

BCS	Bovine Calf Serum
BPH	Benign Prostate Hyperplasia
cDNA	Complementary Deoxyribonucleic Acid
CI	Confidence Interval
CS	Calf Serum
CSD	Casodex
DAPI	4'-6-Diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic Acid
dNTP	Dinucleotide Triphosphate
FACS	Fluorescence-activating cell sorter
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GED	GTPase effector domain
GTP	Guanosine-5'- triphosphate
ICC	Immunocytochemistry
IFN	Interferon

IHC	Immunohistochemistry
ISRE	Interferon Stimulated Response Element
JAK	Janus Kinase
MD	Middle Domain
MX1	Myxovirus (influenza) resistance 1
MxA	Myxovirus (influenza) resistance A
N	Number of samples
NCBI	National Center for Bioinformatics
OAS1	Oligoadenylate synthetase 1
OR	Odds ratio
PBS	Phosphate Buffered Saline
PCa	Prostate Cancer
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PIA	Proliferative Inflammatory Atrophy
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate Specific Antigen
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RR	Risk Ratio

SEM	Standard error of the mean
SNP	Single Nucleotide Polymorphism
STAT1	Signal transducer and activation of transcription 1
STI	Sexually Transmitted Infection
XMRV	Xenotropic murine leukemia-related virus

CHAPTER 1

INTRODUCTION

The link between cancer and viruses has been one of the pivotal discoveries in cancer research where viruses have been estimated to be involved in approximately 10-15% of all cancers. Recent studies provide experimental and epidemiological data that have correlated prostatitis and viral and/or bacterial infections with increased PCa risk. Results are consistent with the hypothesis that the anti-viral pathways could be major determinants of cancer initiation. Most, if not all, anti-viral pathways are regulated by interferons (IFN). IFN-induced pathways are significantly down-regulated in cancers. Evidence that viral pathways are de-regulated in prostate cancer comes from the studies demonstrating that RNASE L is de-regulated/ mutated in hereditary prostate cancer. Our lab showed that androgens primarily regulate the immune inflammatory pathways in normal prostate epithelial cells including MxA. Myxovirus (influenza virus) resistance A (MxA), is an interferon regulated antiviral protein. MxA may have a role in prostate cancer initiation by possibly interfering with viral infection in pre-initiation stages of PCa. Our broad hypothesis is that MxA expression is associated with prostate cancer (i.e. the viral response pathway is not functional in prostate cancer). In searching for evidence to support our hypothesis we determined that MxA expression was decreased in prostate cancer (NCBI- GEO Database). This evidence establishes a clinical association/link between MxA and prostate cancer. The objective of the current project was to determine

the expression/function of MxA and to understand its molecular mechanism of action in prostate cancer. We addressed this objective with the following specific aims:

1. Investigate the association of the MxA Polymorphism (rs2071430) with prostate cancer.
2. Determine the mechanism by which MxA is regulated in prostate cancer and its functional significance.
3. Elucidate the functional significance of MxA on the cancer phenotype (loss/gain of MxA expression)

CHAPTER 2

LITERATURE REVIEW

2.1 Prostate Cancer

Prostate cancer is the second most common type of cancer among men in the United States. According to American Cancer Society there will be an estimated 238,590 new cases of prostate cancer in 2013 and 29,720 deaths, which is a significant increase since 2009 (1).

The prostate is an exocrine gland of the male reproductive system that is located directly under the bladder and in front of the rectum as illustrated in Appendix Figure 1. Prostate cancer is an androgen-driven cancer which is a trait that can be used both for screening purposes and as a target for early therapies. Early prostate cancer grows locally within the prostate, often for many years. Eventually, prostate cancer extends outside the prostate, and spreads in three ways (i) by invading into neighboring tissues (ii) by spreading through the lymph system of lymph nodes and lymph vessels and (iii) by traveling to distant tissues through the blood (metastasis) (2).

There are four distinct stages of prostate cancer as seen in Appendix Figure 2. In stage I, prostate cancer is confined only in the prostate gland area. Stage I prostate cancer is microscopic and difficult to diagnose (2). In stage II, the prostate tumor has grown inside the prostate but hasn't yet extended beyond it. Stage III prostate cancer has spread outside of the prostate minimally. Prostate cancer in stage III may involve nearby tissues

(1). In stage IV, the cancer has metastasized outside of the prostate to other tissues. Stage IV prostate cancer commonly spreads to lymph nodes, the bones, liver, or lungs. Prostate cancer stage helps determine the optimal treatment, as well as prognosis (2). Accurately identifying the prostate cancer stage is extremely important and one of the most common ways to screen and diagnose prostate cancer is by PSA screening.

Prostate specific antigen (PSA) is a glycoprotein that is produced by the prostatic epithelium (3). An increase in PSA can be an indication of abnormal prostatic growth and is used in some countries as a non-invasive means of screening for prostate cancer; normally a threshold of 4 ng/ml is used (4). The concentration of PSA that is considered to be normal depends greatly on the age of the patient as well. There are several controversies regarding screening for prostate cancer using PSA, and the screening programs vary greatly from country to country (5, 6).

For diagnostic purposes, regular PSA measurements are used in conjunction with digital rectal examination and trans-rectal ultrasound guided biopsy of the prostate (7). The introduction of PSA screening programs in the late 1980s lead to a dramatic increase in the number of cases diagnosed annually, especially of patients with a low-risk cancer (8). This has meant a shift from diagnosing mainly high risk, late stage prostate cancer to detecting low-risk disease, which of course improves the chances of successful treatment. The primary concern is over diagnosis, which is leading to over treatment of cancer that would not become symptomatic (9). Treatment of prostate cancer depends on the stage of the diagnosed tumor and of the age and life expectancy of the patient. Treatment for prostate cancer can be: radical prostatectomy, radiotherapy, hormonal therapy or a combination of them (1) .

The exact cause of prostate cancer is not yet known but there are certain risk factors linked to the disease. Numerous studies have been conducted on the genetic etiology of the disease. There is emerging evidence that prostate inflammation may contribute to prostatic carcinogenesis. Evidence from genetic and molecular studies also supports the hypothesis that prostate inflammation and/or infection may be a cause of prostate cancer (10). Epidemiological data have correlated prostatitis, sexually transmitted diseases, as well as viral and bacterial infections with increased risk of prostate cancer (11, 12).

2.1.1 Prostate cancer and genetic factors

Over the past years multiple pieces of evidence support the notion that genetics plays a role in prostate cancer susceptibility and pathogenesis. The evidence also indicates that the genetic basis of prostate cancer is far more complex than what was initially anticipated. To study the link between prostate cancer (PCa) and genetics; large families have been studied in which prostate cancer clusters together. Early observations were made in large families studied in Utah, but the first description of familial aggregation of prostate cancer was reported as far back as 1956 (13).

Several published studies have illustrated an increased risk for prostate cancer among men with a family history for prostate cancer. In 2003, Bruner et al. (14) published a meta-analysis of 16 case-control studies and eight cohort studies supporting this previous theory. The term “family history” was defined as cases where a father, brother, any first- or second-degree relative or other relative had been affected by prostate cancer (14). Pooled Risk ratios (RRs) for men with any affected family member, one affected first-degree relative and one affected second-degree relative were 1.9 (95% CI,

1.6-2.2), 2.2 (95% CI, 2.1-2.4) and 1.9 (95% CI, 1.6-2.3), respectively. These results illustrate familial risk of prostate cancer. Further increases in risk ratios (RR) were also consistently observed when (i) more than one relative was affected, (ii) the closeness of the affected relative to the unaffected individual increased, and (iii) the age of onset of the affected decreased. Taken together, this is strong evidence for a genetic component in familial prostate disease.

Although more than 40% of prostate cancer cases are estimated to be an effect of genetic variation (15), rare, highly penetrant genes probably account for a minority of prostate cancer cases. Mutations in high-penetrance susceptibility genes increase the risk of cancer several fold, and tumors with such mutations are often called hereditary cancers. So far, there have been high-risk candidate genes identified to include; HPC/ELAC2 on 17p, MSR1 on 8p22-23, and RNASEL on 1q25 (16-18).

RNASEL is a constitutively expressed latent endoribonuclease that mediates the antiviral and pro-apoptotic activities of the interferon inducible 2-5A system (19). Once activated by interferon, cells containing a functional RNASEL gene produce an enzyme that degrades single-stranded RNA, leading to apoptosis (20). Defects in the RNASEL gene have been shown to result in reduced immunity to viral infections and cancer (21). A genome-wide scan for linkage in prostate cancer families found evidence for a prostate cancer susceptibility locus on chromosome 1q24-25 (22). In 2002, Cartpen et al. identified RNASEL as a candidate gene for the HPC1 locus through a positional cloning and candidate approach. A truncating mutation (E265X) and an initiation codon mutation (M1I) were reported to co-segregate within two specific prostate cancer families (16). In

addition to the rare mutations, a number of relatively common RNASEL variants have been associated with prostate cancer risk.

There is some confirmatory evidence for the association between variants of RNASEL and prostate cancer risk (23), but other studies have shown no association (24-26). In summary, the role of RNASEL in the pathogenesis of prostate cancer is still unclear, due to inconsistencies in the association studies. However, reported in a review from 2004, "it is tempting to speculate that genetic variants of the RNASEL gene increase the risk for prostate cancer only in the presence of some environmental factor (e.g. viral infection) that may vary between different study populations" (27).

2.1.2 Polymorphism and prostate cancer

The remainder of the genetic influence is most likely mediated by more common genetic variants or polymorphisms. Low penetrance polymorphisms increase the risk of cancer only modestly, but the prevalence of such polymorphisms may be higher than the prevalence of mutations in high penetrance susceptibility genes, and thus their overall existence could prove to be substantial (28). The list of these variants is long, but the major pathways currently under investigation include those involved in DNA repair (29-31), carcinogen metabolism, the action of androgens (28, 32) as well as inflammation pathways (33-35). Numerous polymorphisms have already been suggested to be associated with the risk of prostate cancer one in particular being a part of the inflammation pathway. One approach is to elucidate whether functional polymorphisms in genes that regulate inflammatory processes, such as cytokines, confer altered risks for developing cancer or are potential prognostic factors.

2.2 Inflammation and cancer

Various carcinomas (including cancers of the liver, bladder, colon, stomach, and esophagus) have been shown to arise from areas of infection and inflammation. Over 15% of all malignancies worldwide are attributable to infectious agents, and inflammation is a major component of these chronic infections (36). Colon cancers arising in individuals with inflammatory bowel disease (e.g. chronic ulcerative colitis or Crohn's disease) and stomach cancers caused by chronic *Helicobacter pylori* infection are among the most intensively studied and well established types of cancer associated with inflammation of different origins (37).

Inflammation involves the induction of complex, coordinated chemical signals and associated physiological processes following injury that promote "healing" of damaged tissues. Early responses include increases in vascular permeability and activation, together with the directed migration of leukocytes towards the site of injury. This is where the ground-work is laid for the formation of a new extracellular matrix. The directional migration of leukocytes is mediated by secreted chemokines that form a concentration gradient towards the site of inflammation. The extracellular matrix provides the structure upon which cells can migrate, proliferate, and regenerate new tissue and a vascular network. In the later stage of the inflammatory response, the macrophages are the dominant cell type, orchestrating and directing the healing process. Normally, inflammation is a self-limiting process due to the production of anti-inflammatory cytokines that buffer the effect of pro-inflammatory cytokines. The cytokine/chemokine pattern persisting at the inflammatory site is important in the

development of chronic disease. Dysregulation of any of the cooperating factors can lead to prolonged inflammation with chronic exposure to cytotoxic mediators (37).

Chronic inflammation can be caused by a variety of factors, including bacterial, viral, and parasitic infections, chemical irritants, and non-digestible particles. Oftentimes the underlying cause of chronic inflammation is not known. The longer the inflammation persists, the higher the risk of associated carcinogenesis (38). At the site of inflammation, caused by either wounding or infection, phagocytic cells (e.g. neutrophils and macrophages) generate reactive oxygen and nitrogen substances, but these cells also synthesize and secrete large quantities of growth factors and a number of potent angiogenic factors, cytokines, and proteases, all of which are important mediators in the tissue regeneration, but can also potentiate neoplastic tumorigenesis.

2.2.1 Inflammation and prostate cancer

Although it has been established that chronic inflammation plays a causative role in the development of many human cancers, the contribution of inflammatory processes to the development of prostate cancer has not been extensively studied. However, data from epidemiological, genetic and molecular pathology studies have been accumulating recently and suggest that inflammatory processes are also involved in the development of prostate cancer. The chronic inflammatory microenvironment, characterized by the accumulation of macrophages and lymphocytes, is extremely common in the tissue stroma and epithelium of the prostate and may support both the initiation and progression of prostate cancer (39).

Due to the combined findings of high proliferation indices and inflammatory infiltrations seen in prostate lesions, the term proliferative inflammatory atrophy, or PIA,

was introduced (40). The underlying causes of PIA lesions are still not clearly understood, but they may arise either as a consequence of epithelial damage (from infection, ischemia, or toxin exposure) or as a direct consequence of inflammatory oxidant damage to the epithelium (41). Accumulating evidence from morphological, immunohistochemical and genetic studies have provided evidence for the concept that PIA lesions may be precursors to prostate cancer, partly because the vast majority of such lesions are found adjacent to or near early adenocarcinomas or high grade prostatic intraepithelial neoplasms, or both (40, 42, 43). Further support for the hypothesis comes from the fact that PIA shares several molecular alterations found in both prostate intraepithelial neoplasia (PIN) and prostate cancer (40).

In addition, chromosome 8 abnormalities, which are common occurrences in prostate cancer, were found in areas of PIA lesions. Acquired chromosome 8 abnormalities have been noted in about 1% of normal prostate tissue, 4% of PIA lesions, and about 6% of adenocarcinomas (44). Other studies have reported p53 mutations in 5% of PIA lesions, a rate similar to that seen in high-grade PIN, compared to 20% of prostate cancer cases (45). Thus, these are genetic changes that are associated with PIN and prostate cancer. Even though PIA lesions may be an early histological precursor to prostate cancer, it is important to note that some studies have not found an association between PIA and prostate cancer (46). Moreover, not all cancers occur within or in the vicinity of PIA. In fact, PIA may simply indicate an intraprostatic environment that favors and leads to prostate cancer development as seen in Figure 3.

2.2.2 Prostatitis and prostate cancer

Prostatitis, which is the inflammation of the prostate, is a common urological disorder worldwide; 2-10% of men experience it during their lifetime (47). The clinical presentation is characterized by uncomfortable symptoms, such as dysuria, and rectal and suprapubic pain (48). Prostatitis is classified into four categories: I, acute bacterial; II, chronic bacterial; III, chronic nonbacterial/chronic pelvic pain syndrome; and IV, asymptomatic prostatitis. Both acute bacterial prostatitis (which is usually caused by *Escherichia coli*) and chronic bacterial prostatitis (caused by *E. coli* and several other infectious agents) are normally treated with antibiotics. Chronic non-bacterial prostatitis/chronic pelvic pain syndrome, is the most common form, affecting 90-95% of patients with prostatitis. Men with this type of prostatitis may experience symptoms for weeks to years, and current therapy has limited success in alleviating the pain that the patients experience. Asymptomatic prostatitis is a histological diagnosis of prostate inflammation that is found subsequent to pathological examination of the prostate tissue. This category is found in a significant number of patients and epidemiologic studies have estimated its prevalence to be as high as 32.2% in a population of men with elevated Prostate Specific Antigen (PSA) (49). The PSA test has been widely used to screen men for prostate cancer. It is also used to monitor men who have been diagnosed with prostate cancer to see if their cancer has recurred after initial treatment or is responding to therapy (3).

Several case-control studies have investigated the association between prostate cancer and prostatitis, with variable results. In 2002, Dennis et al. performed a meta-analysis of 11 case-control studies that evaluated the possible relationship between

prostatitis and prostate cancer. The meta-analysis found an increased risk of prostate cancer among men with a history of clinical, or symptomatic prostatitis (OR=1.6; 95% CI, 1.0-2.4), particularly in population-based case-control studies (OR=1.8; 95% CI, 1.1-3.0) (50). There are numerous complexities involved in diagnosing prostatitis and the risks for potential recall or detection bias when performing association studies. Those complexities in the association between prostatitis and prostate cancer make the analyses problematic. The results could reflect an effect of detection bias, since patients with prostatitis may be more likely to be monitored by a urologist and thus more likely to be screened for prostate cancer, or the results could reflect a recall bias, because patients with prostate cancer may try to remember more about potential exposure to prostatitis than the controls (50).

Epidemiological studies investigating the possible association between prostatitis and prostate cancer have relied on patient reports for information on prostatitis history. Therefore, there are serious problems in using studies of this type to identify valid relationships between category IV prostatitis and prostate cancer. The reason why inflammation leads to symptoms in some men but not others is not known. It is possible that a distinguishing feature between symptomatic and asymptomatic prostatitis may be the anatomical localization of the prostatic inflammation. Inflammation in the peri-urethral region, or transition zone, may be more likely to manifest in urinary symptoms and pain than inflammation in the outer areas of the gland, or the peripheral zone, where prostate cancer more commonly develops (27).

Two epidemiological studies have illustrated that the prevalence of prostatitis correlates with the prevalence of prostate cancer worldwide, with rates of prostatitis and prostate cancer being much higher in North America than in Asia (48, 51).

Sexually transmitted infections (STIs) have also long been hypothesized to increase the risk for prostatitis and prostate cancer. STIs are theorized to increase the risk of prostate cancer by causing inflammation of the prostate, which may in turn lead to the initiation of carcinogenesis (52). These STIs often times are viral in nature.

2.2.3 Viruses and cancer

Because it was shown that nearly 15-25% of cancers have a viral etiology, it is important to understand the association between viruses, anti-viral pathways and cancer (53). Several viruses have been linked to many types of cancer. The Epstein-Barr virus has been linked with Burkitt's lymphoma (54). The Hepatitis B and C viruses have been linked with liver cancer in people with chronic infections. HTLV-I, a retrovirus, has been linked to T-cell leukemia (55) illustrated in Figure 4. Papilloma viruses have been linked with cervical cancer and skin cancer; however, five studies investigating possible associations between human papilloma virus (HPV) and prostate cancer have found variable results. The results range from no association to statistically significantly higher risks for HPV-seropositive men (27).

A meta-analysis study by Taylor et al. also examined the associations between HPV infection and prostate cancer risk detected in ten studies using either serological or PCR based evidence of HPV infections. The results showed an odds ratio (OR) of 1.39 (95% CI, 1.13-1.71). Notably, while HPV is known to be an oncogenic virus, its influence on prostate carcinogenesis may be independent of inflammation.

Several studies have investigated the presence of infectious agents in prostate tissue. Various viruses and bacteria were found, but the results have not been very consistent, possibly due to methodological differences (such as differences in the tissue handling and detection methods), or possible contamination from agents in areas close to the prostate (56). It is also worth noting that STIs detected in the prostate cancer tissues could have been acquired after the initiation of carcinogenesis. Furthermore, the failure to detect infectious agents in the prostate tissue does not necessarily mean that these agents do not play a role in prostate carcinogenesis, as some may be cleared after causing damage (41). Human papilloma viruses, human herpes virus-8, Herpes simplex virus-2, cytomegalovirus and Epstein-Barr virus are some of the agents that have been found in prostate tissue(27).

Xenotropic murine leukemia virus-related virus (XMRV) was previously discovered in a subset of men with prostate cancer and was thought to be the first gammaretrovirus known to infect humans (19); however, researchers found that XMRV replicated easily in cultured human prostate tissue, but not in other types of human tissues. There was an association of this virus with malignant prostatic tissues (57); however, it has been established that virus detected was actually contracted through RNA that had been contaminated with cells which produce high titer of the XMRV virus. 22Rv1 cells were derived from serial passage through nude mice xenographs (56, 58). Discovery of XMRV followed investigations of the role of the antiviral enzyme RNase L in hereditary prostate cancer. A R462Q certain mutation in RNASEL is associated with increased XMRV viral infection in prostate cancer (19). RNase L is required for a complete interferon (IFN) antiviral response against XMRV as well as other viruses.

The immune system protects the organism from diseases by identifying and killing pathogens or tumor cells. Pathogens that can be detected range from viruses to multicellular parasitic worms. According to the functional mechanism and effects, immunity can be classified into two types, innate immunity and adaptive immunity (59). The innate immunity provides non-specific defenses against pathogenic challenges but does not confer long-lasting effects to these protections, while adaptive immunity, activated by the innate immune system, generates highly specialized cells and systematic processes that eliminate invading pathogens and exhibit a memory response upon reinfection (59).

Recently a third form of immunity was discovered and termed 'intrinsic immunity'. It is characterized by constant expression of genetically coded proteins specifically targeted against eukaryotic retroviruses (60, 61). A major function of the vertebrate innate immune system is the recruitment of certain immune cells to infection sites through specialized small proteins called cytokines/chemokines, which act as the signaling mediators between the cells (59).

2.3 Interferons/Antiviral Pathways

The interferon (IFN) system plays an essential role in the first line of defense against inflammation and viruses. Many cell types produce and secrete IFN alpha and IFN beta in response to many viral infections (62) as seen in Figure 5. This signals the presence of an invading virus and acts in a paracrine manner as a warning signal to neighboring cells. There are many types of interferons depicted in Figure 6. Our focus is mainly on Type I IFN. These interferons bind to the IFNAR1 receptors. The binding of Type I IFN to this receptor complex triggers the intracellular Jak/STAT pathway.

Activation of the JAK/STAT signaling pathway constitutes an important regulatory mechanism by which host cells inhibit viral infections. The central transcriptional activators of this pathway are the STAT proteins. The STAT-1, STAT-2, and STAT-3 proteins are important regulators of the innate immune responses to infections by a variety of RNA and DNA viruses (63-66). The activation of this signaling cascade then leads to the activation and enhanced expression of more than 300 known genes (67-69). These different actions converge to produce the “antiviral state/response”. There are three major antiviral pathways, OAS, PKR, and MxA that are activated to produce an antiviral state as depicted in Figure 7.

Overall, results suggest that the anti-viral pathways (interferon) could be major determinants of cancer initiation. Dysfunction of the immune system has been documented in many types of cancers to include melanoma (70). The precise nature and molecular basis of immune dysfunction in cancer is not well defined. Defects in interferon signaling represent mechanisms of immune dysfunction in cancer (70). Cancer inhibits the immune system by various cellular and molecular mechanisms.

Dysfunction of the immune system arises during the early stages of cancer and throughout progression to metastatic disease (71). There are indications that suggest that normally, the interferon pathway is important in fighting off cancers because of studies done on mouse models in which the pathway was purposely disrupted. In those studies the mice developed spontaneous tumors at higher rates than in normal mice with functional interferon signaling. This evidence suggests that the immune system is able to stop many cancers when they initially begin and the interferon pathway may harbor a general immune defect in many types of cancer (72).

2.3.1 Interferons

Among the cytokines, interferons (IFNs) are the earliest discovered examples and represent a large family of proteins exhibiting strong antiviral functions (73-75). The IFN family is mainly comprised of two types of related cytokines: Type I IFNs and Type II IFN (76). The Type I group consists of 7 classes, IFN- α , IFN- β , IFN ϵ , IFN- κ , IFN- ω , IFN- δ , and IFN- τ . IFN- α has 13 subspecies and other IFNs only have one (77-79). IFN- α , IFN- β , IFN- ϵ , IFN- κ and IFN- ω are found in humans whereas IFN- δ and IFN- τ are described only in pigs and cattle, respectively (75). Type II IFN contains only IFN- γ . Type I and Type II IFNs are structurally distinct: they bind different receptors and are encoded by separate chromosomal loci. Genes encoding Type I IFNs are clustered on human chromosome 9 and Type II IFN genes are located on chromosome 12 in humans (74).

The most prevalent interferon present in epidermal keratinocytes is IFN kappa. IFN kappa also belongs to the Type I IFN and displays approximately 30 % homology to other members of the interferon family (78). It has been reported that IFN-kappa signals through the canonical type 1 interferon receptor complex (consisting of the two subunits IFN- α R1 and IFN- α R2) (80).

In recent years, another class of IFN-like molecules have been discovered and named IFN- λ 1, IFN- λ 2 and IFN- λ 3 (also known as interleukin (IL)-29, IL28A and IL28B) (81, 82). Genes encoding IFN- λ s are clustered on human chromosome 19 (82). IFN- λ s have a similar but independent functional mechanism from Type I IFNs and are sometimes referred as Type III interferons (81-83).

In the IFN family, IFN- α , IFN- β and IFN- γ are currently the most well-characterized members. IFN- α , IFN- β , IFN- κ and IFN- λ are the mediators for the expression of Mx proteins. Therefore these Type I IFNs will be discussed in the following sections.

2.3.2 Interferon functions

Type I IFNs are expressed at a low level in almost all cell types, although hematopoietic cells are the main producers of IFN- α while IFN- β is mostly secreted in fibroblasts (IFN- α and IFN- β were therefore originally termed leukocyte and fibroblast IFN, respectively) (84). Generally speaking, viruses or double-stranded (ds) RNA can induce human cells to produce IFN- α and IFN- β , and the ratio of the expressed IFNs varies by the organism, tissue, and viral species. IFN- α and IFN- β are mainly involved in innate immune response against viral infection. Some IFN- α species also exhibit other functions such as anti-proliferation and stimulation of cytotoxic activities of natural killer (NK) cells and T cells and up-regulation of major histocompatibility complex (MHC) class I antigen expression in cells (74, 82, 85).

Major production of IFN- γ takes place in CD4⁺ T helper cell type 1 (Th1) lymphocytes and CD8⁺ cytotoxic lymphocytes when these cells are stimulated with specific antigens or mitogens such as staphylococcal enterotoxin A or B, during the adaptive immune response (84, 86). IFN- γ is also secreted by NK cells, B cells, NKT cells and possibly professional antigen-presenting cells (APCs) (87). The most distinguishing feature of IFN- γ from other IFNs is its versatile immunomodulatory activity, although IFN- γ is also involved in the immediate cellular response to viral

infections. This activity directly affect Th1-type response development, B cell promotion and local leukocyte endothelial interactions (88). Other important functions of IFN- γ include macrophage stimulation, NK cell promotion and up-regulation of the Class I & II antigen presentation pathways in normal cells (89-91).

As a relatively new member of the IFN family, IFN- λ has not yet been intensively studied. It is typically co-expressed with type I IFNs by virus infected cells and has antiviral activity against certain viruses that may, or may not, be suppressed by other Type I IFNs, but in a similar way as Type I IFNs (74, 81, 92, 93).

2.3.3 Induction of interferon expression

The induction of IFN expression is regulated at the transcriptional level. For type I IFNs, the best understood example for regulation is IFN- β . The IFN- β gene (IFNB) promoter can interact with several transcription factors that cooperatively enhance the IFNB transcription level (83). Of these transcription factors, interferon regulatory factor 3 (IRF-3) is thought to play the central role (94). Phosphorylation is required for IRF-3 activation.

Phosphorylated IRF-3 subsequently dimerizes and translocates into the nucleus to cooperate with the transcriptional activator CREB-binding protein (CBP) and initiate IFNB transcription. Another IRF family member, IRF-7, is also an important enhancer which is utilized by the majority of INF- α promoters and is absolutely essential for IFN- α expression. Unlike the constitutive expression of IRF-3, IRF-7 gene is IFN inducible (95). Therefore, IFNB is transcribed in the early response, whereas IFN- α species are expressed in relatively higher amounts but with delayed kinetics (95, 96). Two essential partners of IRFs, nuclear factor κ B (NF- κ B) and activated protein-1 (AP-1, ATF-2/cJun

particularly), are promoted as a more general stress response. They work together to enhance IFNB transcription (83).

Induction of IFN- γ expression appears to be more complex, as different pathways are found in distinct cell types. On the cellular level, IFN- γ production can be stimulated by cytokines secreted by APCs, mostly IL-12 and IL-18, and negatively regulated by IL-4, IL-10, transforming growth factor- β (TGF- β) and glucocorticoids (97-102). On the genetic level, transcription factors such as YY1, nuclear factor of activated T cells (NFAT), AP-1, NF- κ B and signal transducer and activator of transcription proteins (STATs) are found to bind the IFN- γ gene (IFNG) promoter regions (103-108). Like type I IFNs, positive feedback effect also exists as an important up-regulatory means in IFN- γ induction (87).

The induction of IFN- λ expression is overall similar to that of Type I IFNs. IFN- λ gene transcription is also controlled by the IRF and NF- κ B pathways when viral or other PAMPs are present (109). It was also shown that IFN- λ 1 induction is largely dependent on IRF-3 as with IFNB, whereas IFN- λ 2/3 expression mainly relies on IRF-7 as in the case of INF- α . Moreover, IFN- λ 2/3 has delayed kinetics in comparison to IFN- λ 1 in the immune response, which is analogous to the later response of INF- α compared to INF- β (110).

Despite these similarities, however, the induction of IFN- λ s has also been reported to possess substantial differences from that of Type I IFNs. Firstly, IFN- λ s and Type I IFNs are not always expressed in the same cell types. Exceptions are found in macrophages which express Type I IFNs but not IFN- λ 1 in response to influenza A virus, while under the same conditions, human alveolar type II epithelial cells produce IFN- λ 1

but not IFN- β (111). Secondly, distinct from the cooperative manner of transcription factors in the Type I IFN promoter activation, IRF and NF- κ B regulate IFN- λ 1 induction independently, as the binding sites for IRF and NF- κ B have been found spatially separated on the IFN- λ 1 gene promoter (112). In addition, NF- κ B seems to be the key controller of IFN- λ production, rather than IRFs that play dominant role in IFN- α/β expression (113).

2.3.4 Interferon-induced signaling pathways

In the immune response, secreted IFNs bind membrane receptors and initiate signal transduction pathways that result in the expression of numerous IFN-stimulating genes (ISGs). Type I, II and III IFNs bind to distinct membrane receptor complexes as illustrated in Figure 9. Each one of the receptor chains interacts with a member of the Janus activated kinase (Jak) family (114, 115). The Jak-STAT pathway was the first signaling pathway shown to be activated by IFNs (116-119). The models of the IFN-mediated signaling mechanism via this pathway have been well established.

In the case of Type I IFNs, the receptor is composed of two subunits, IFN- α R1 and IFN- α R2 (120). IFN- α R1 and IFN- α R2 are constitutively associated with tyrosine kinase 2 (Tyk2) and Jak1, respectively. When a monomeric Type I IFN is bound (121), the receptor chains undergo a structural rearrangement and their associated Jaks are rapidly autophosphorylated (119). In a major pathway for Type I IFNs, activated receptor complexes subsequently regulate the phosphorylation of STAT1 and STAT2. Phosphorylated STAT1 and STAT2 dimerize and recruit a non-phosphorylated factor IRF-9 to form a heterotrimer called ISG factor 3 (ISGF3) (122), which enters the nucleus and binds to IFN-stimulated response elements (ISRE) to initiate the transcription of

certain ISGs demonstrated in Figure 8 such as MxA, IRF3 and OAS1 that possess this promoter (123).

Besides ISGF3, activated Type I IFN receptor is also responsible for the activation and homo- or heterodimer formation of other STATs (114, 124). While ISGF3 is the only complex that binds ISRE, these activated STAT homodimers bind another type of element known as IFN- γ activated site (125) elements and thereby induce the transcription of GAS-presenting ISGs (126, 127). It has been proposed that different combination of STATs may be induced to target the transcription of specific genes with distinct functions (75). Of the many genes that are induced, the gene of our interest belongs to the distinct dynamin superfamily.

2.4 Overview of the dynamin superfamily

The dynamin superfamily comprises a class of guanine-nucleotide-binding (G) proteins, or GTPases (hydrolases that catalyze the hydrolysis of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) and phosphate). These proteins possess essential functions in cellular processes relying on membrane remodeling (128). In addition to classical dynamins, an increasing number of dynamin-related proteins have been assigned to this superfamily (Fig. 9), such as the Mx proteins (129), the GBP/atlastin family (130), the bacterial dynamin-like proteins (BDLPs) (131) and the EHD ATPases (132). All members share common biochemical properties and have (i) low binding affinity to GTP, (ii) the propensity to self-assemble and oligomerize around lipid templates and (iii) increased nucleotide hydrolysis rates promoted by oligomerization (133, 134).

Because of these features, the dynamin superfamily of large GTPases are distinguished from the small Ras-like and other regulatory GTPases, such as the α -

subunits of heterotrimeric G-proteins and the translation factors in protein synthesis (128, 135-138). These distinct biochemical features of dynamin superfamily members can be explained by their special multi-domain architecture. Apart from a large N-terminal GTPase (G) domain containing around 300 amino acid residues, the members usually also have two additional domains: the so-called middle domain (2) and the GTPase effector domain (GED) that were thought to be crucial to the oligomerization and GTPase activity stimulation at least in some members, such as dynamins and Mx proteins (Fig.10) (139-142). Different members within the superfamily also carry other domains individually for their specific cellular functions (Fig. 11) (128).

Primary sequence analysis indicates that the G domain of dynamin superfamily members is an extended form of the canonical GTPase fold observed in Ras and many other GTPases, as the four essential motifs, sequentially named G1-G4, are all present. All these motifs contribute to the binding and hydrolysis of GTP, and they are hallmarked by one or more conserved amino acid residues (143). The G1 motif has the consensus sequence of GXXXXGKS/T ("X" stands for an unspecific residue) and forms the conserved phosphate-binding (144) loop, which is responsible for the interaction with the β - and γ -phosphate of the nucleotide (145). The G2 motif contains an invariant threonine residue that binds the γ -phosphate of GTP (135). The G3 motif has a DXXG composition where the conserved aspartate binds a catalytic magnesium ion (Mg^{2+}) and the glycine coordinates γ -phosphate (143). The G4 motif has a T/NKXD signature (except for GBPs and OPAs) that is involved in the coordination of the nucleotide base and the ribose (135). Two conserved loop regions in the G domain were named switch I and II, as they undergo large conformational changes in the GDP- and GTP-bound form in analogy to

the switch that mediates the "on" and "off" state of the molecule (146). Switch I covers the G2 motif and the switch II overlaps with the G3 motif.

In contrast to many molecular structures of the G domain, little structural information of the much less conserved MD and GED of the main dynamin superfamily members (such as dynamins, DLPs or Mx proteins) is known. According to secondary structure prediction based on primary amino acid sequence, MD and GED fold predominantly into α -helices (147), which was also observed for some far relatives of dynamins and Mx proteins (130, 131).

2.4.1 Mx proteins

Mx proteins are key mediators of the IFN-induced innate antiviral response in vertebrates (129). Their discovery dates back on genetic studies on inborn resistance of mice to influenza viruses about 40 years ago (148, 149). Mx1 protein was cloned from influenza virus resistant mice (150) and was shown to mediate cell intrinsic antiviral activity (151). The mouse Mx1 is encoded by the Mx1 gene on chromosome 16 and most influenza virus-susceptible mouse strains carry large deletions or nonsense mutations in this region (152).

Later it was found in the mouse genome that another gene, named Mx2, which is closely linked with Mx1 on chromosome 16, is also defective in inbred mouse strains (153). Human Mx proteins were identified from cross-interactions of a monoclonal antibody (2C12) against mouse Mx1 in human cells (154). Subsequently, two proteins named MxA and MxB were found to be encoded by closely linked genes on human chromosome 21 (155-157). Subsequently, IFN-inducible Mx genes were identified in

many vertebrates and their encoding proteins can be classified into several subgroups based on sequence similarities (Fig. 11) (129).

Mx proteins have a molecular mass of around 75 kDa, and are composed of the N-terminal G domain, the MD and the C-terminal GED as described in the dynamin superfamily. The G domain of Mx proteins share 40% sequence identity to other dynamin superfamily members whereas for MD and GED only 20% identity is observed (158). Compared to dynamin, Mx proteins usually have an extra unstructured N-terminal stretch of amino acids with unknown function, and this stretch varies in the length and sequence of amino acid residues for Mx proteins from different species.

In solution, Mx proteins self-assemble into high-order oligomers in ring-like or helical arrangement (159, 160). In living cells, rodent Mx proteins accumulate in distinct nuclear dots near promyelocytic leukemia (PML) nuclear bodies (NBs) in IFN-treated cells (161), while human MxA forms punctuate granula in the cytoplasm and partially co-localizes with COPI positive membranes of the smooth ER-Golgi intermediate compartment (162). It was concluded that these Mx assemblies may serve as an intracellular storage form from where more Mx proteins can be recruited when needed (163).

Mx proteins have different specificities in their antiviral spectrum and this may be a result of their subcellular localizations to some extent. For example, rodent Mx1 has a C-terminal nuclear localization signal (NLS) and inhibits multiplication of orthomyxoviruses, such as influenza virus (FLUAV) and Thogoto virus (THOV) which replicate in the cell nucleus (129). In contrast, cytoplasmic rodent Mx2 confers resistance to those viruses that replicate in the cytoplasm, such as rhabdoviruses (e.g. vesicular

stomatitis virus, VSV) and bunyaviruses (e.g. LaCrosse virus, LACV) (163). On the other hand, cytoplasmic human MxA is able to suppress a broad range of different virus classes, including members of the bunyaviruses in Figure 12, orthomyxoviruses, paramyxoviruses, rhabdoviruses, toga-viruses, picornaviruses, reoviruses and hepatitis B virus (HBV), a DNA virus with a genomic RNA intermediate, despite their different replication sites in the cell (129, 163).

Recently, it was reported that human MxA can also suppress a large dsDNA virus named African swine fever virus (ASFV) (164); however, nucleus-localizing human MxB has no apparent antiviral activities (165). As for avians, chicken Mx proteins also lack the ability to fight against avian influenza viruses (166). In the case of fish Mx proteins, Atlantic salmon Mx1 was reported to be able to inhibit the activity of an influenza-like fish virus named infectious salmon anaemia virus (ISAV) and an aquatic birnavirus called infectious pancreatic necrosis virus (IPNV) (167).

From early transgenic mouse and other cell-based assays, it was demonstrated that both mouse and human Mx proteins possess intrinsic antiviral activity *in vivo* and are able to function autonomously in the absence of other type I IFN-induced factors (129). There have been increasing amounts of research carried out in order to elucidate their antiviral mechanism. It has been suggested that Mx proteins associate with essential viral components and thereby block their functions. For example, human MxA was shown to interact with THOV and LACV NP *in vitro* in a co-sedimentation assay (168). This interaction was also observed in a so-called mini replicon assay that mimics the *in vivo* viral replication with a minimum viral genome. Based on these studies, it was suggested

that MxA does not recognize unassembled viral components but rather assembled nucleocapsids (169).

Further studies revealed that human MxA binds to the NP of bunyaviruses and forms MxA/NP copolymers residing in the smooth ER-Golgi intermediate compartment, which would lead to a depletion of NP at the replication sites of the virus (170). Based on these results, it has been proposed that Mx proteins interfere with the intracellular allocation of viral components by missorting them (129). However, as the relevant structural information is lacking, the antiviral mechanism for Mx proteins on the molecular level still remains unclear.

2.4.2 Myxovirus resistance A/Myxovirus resistance 1

Mx proteins are among the few effector proteins of the IFN α/β system with known antiviral activity. They are highly conserved large GTPases with homology to dynamin and have been found in all vertebrates species investigated so far, including mammals, birds, and fish (158). These proteins present a highly conserved N-terminal GTPase domain of ~ 300 amino acids, a central interactive domain of ~ 150 amino acids, and a GTPase effector domain (GED) of ~ 100 amino acids which encompasses two leucine zippers that have the capacity to form α -helices illustrated in Figure 13

The human MX1 gene encodes for the human MxA protein which is a cytoplasmic protein that is rapidly induced in response to acute viral infections (171). Human MxA is a 76-kDa protein that encompasses GTPase activity with a low affinity for GTP and a high GTP turnover rate (172, 173). As to the GTPase activity of purified histidine-tagged human MxA (His-MxA), the GTP turnover was calculated to be 27 min-

1, and the dissociation constant (K_d) of His-MxA to GDP and GTP were measured to be 100 μ M and 20 μ M, respectively (173).

These Mx proteins are abundantly expressed in interferon-treated cells and play a crucial role in the early antiviral defense against certain RNA viruses which has been demonstrated in studies with transgenic mice (174-176). Human MxA is shown to inhibit multiplication of several RNA viruses in various families including ssRNA and dsRNA such as influenza, vesicular stomatitis virus (VSV) (177), measles virus (178), Thogoto virus (179), Bunyavirus (180), and Semliki forest virus (181). MxA has also been shown to confer resistance to some DNA viral genomes. It recognizes the nucleoprotein of the viral nucleocapsid and prevents viral replication by various mechanisms, depending on the replication site. The proposed antiviral mechanism of MxA is illustrated in Figure 14.

Studies with human MxA protein have shown that the GED is able to specifically contact the middle domain, and that interaction is critical to constitute a functional GTPase domain, as well as for oligomerization (182, 183). For MD and GED, a single point mutation at Leu612 to lysine (L612K) of human MxA resulted in a non-oligomerizable form that is defective in GTP hydrolysis and rapidly degraded (182), but still maintains some antiviral activity (140).

Another single point mutation, E645R in human MxA, was shown to have altered specificity and mode of action against vesicular stomatitis virus (VSV) (184).

Furthermore, the region comprising residues 432-471 was shown to be the binding site of antibody 2C12 (185), and residue 564 was found to be a proteinase K cleavage site (186). Based on these experimental results and the knowledge gained from the GBP1 structure (130), a hypothetical domain arrangement was proposed for MxA which contains a

globular G domain with MD and GED folding into an anti-parallel helical bundle (163). Human MxA was shown to bind to negatively charged membranes and form ring-like oligomers that tubulate liposomes in a way similar to dynamin (187, 188).

MxA wraps around the nucleocapsid, hence preventing it from entering the nucleus or translocating from the replication site (189), whereas in other cases it inhibits RNA transcription (190). MxA also plays a role in the context of cancer. Although it is induced by interferons, there is some evidence that nuclear factors also regulate it. Prostate carcinoma cell lines were found to express MxA even without cytokine stimuli, indicating a more complex regulation (191). MxA was also shown to prevent cell motility by interacting with tubulin which requires its GTPase domain. Although it is not essential for mitosis, it also affects membrane remodeling and endocytosis. In addition, several cancer cell lines were demonstrated to express MxA even in the absence of viral infection.

There is also evidence that MxA may play a role in the apoptotic pathway and may be required for the induction of apoptosis. A significantly higher level of caspase-3 activity was detected in wild-type MxA transfected and treated esophageal cancer cells compared to normal cells treated with 5-FU (192). There is evidence, that MxA could be deleted on chromosome 21 due to the possible fusion of TMPRSS2 and ERG which is associated with aggressive, invasive prostate tumors (191). In addition, it was found, that in breast cancer special AT-rich binding protein 1 (SATB1), a global regulator which controls IFN-inducible genes, suppresses MxA (191) by chromatin remodeling (193). Many of the targets of SATB1 are regulated in response to activated Wnt-pathway (194)

which may explain how SATB1 activity promotes tumor growth and metastasis in breast cancer (195).

In primary head and neck squamous cell carcinoma (HNSCC) the MxA gene is hypermethylated (196). It has been reported that the hepatitis B virus (HBV), which leads to liver cancer, core protein is able to directly inhibit transcription of MxA (197). Another study has shown that despite that, MxA is able to inhibit HBV replication (198).

In general, MxA appears to interfere with steps involving viral transcription although the exact mechanism of action is not known or fully understood, but MxA has exhibited various other functions independent of antiviral activity. It could be possible that MxA may have a role in prostate cancer initiation by interfering with viral infection in pre-initiation stages of PCa. It is also possible that MxA could be performing an entirely different function in prostate cancer. There is increasing evidence that Mx proteins are involved in various other processes and according to Muller et al. increased synthesis of Mx proteins may be deleterious to the organism (199). This shows that Mx proteins may have different functions in other species beside the antiviral defense; therefore it is imperative to investigate this protein in prostate cancer.

CHAPTER 3

THE MYXOVIRUS RESISTANCE A (MxA) GENE -88 G>T SINGLE NUCLEOTIDE POLYMORPHISM IS ASSOCIATED WITH PROSTATE CANCER

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3.1 ABSTRACT

Background: Myxovirus (influenza virus) resistance A (MxA) is an interferon stimulated antiviral protein that is required for a complete antiviral response. MxA polymorphism (rs2071430) is located within an Interferon Stimulated Response Element (ISRE) at position -88 in the gene's promoter region, and it has been associated with increased susceptibility to infections and various diseases. In general, the low promoter activity genotype (GG) promotes susceptibility, whereas the high promoter activity genotype (TT) confers protection to Hepatitis C viral infection. MxA's role in prostate cancer is not fully understood. Previous studies have shown that MxA may be a mediator of the effect of IFN on normal and tumor cell motility. MxA may act as a tumor suppressor and the level of expression may be a predictor of metastatic potential. Based on this information, in this study we investigated the association of this functional polymorphism (rs2071430) in MxA with prostate cancer.

Methods: Sample size and power was calculated using the PGA software. Genomic DNA from controls (n=140) and prostate cancer patients (n=164) were used for genotyping SNP rs2071430 on all samples. Statistical analysis was performed using logistic regression model.

Results: A significant association was observed between rs2071430 genotype GG and prostate cancer. Individuals harboring the GG genotype are at an increased risk of prostate cancer. Data stratification reveals that the mutant GT genotype offers some protection against prostate cancer in Caucasians.

Conclusions: MxA SNP rs2071430 GG genotype is significantly associated with prostate cancer irrespective of race. However, data stratification also suggests that the GT genotype is under-represented in Caucasian subjects suggesting its role in protection against prostate cancer in Caucasians. Although MxA is primarily implicated in viral infection, but it may be also be associated with prostate cancer. Recent studies have implicated viral and bacterial infections with increased prostate cancer risk. Expression of the high promoter activity genotype may offer resistance to prostate cancer infection and possibly influence clinical outcomes.

3.2 INTRODUCTION

The human Myxovirus (influenza virus) resistance 1 gene encodes for the human MxA protein (76Kda) a cytoplasmic protein that is rapidly induced in response to acute viral infections (171). MxA belongs to the family of Mx proteins which are among the few effector proteins of the IFN α/β system with known antiviral activity. The Mx proteins are abundantly expressed in interferon-treated cells and play a crucial role in the

early antiviral defense against certain RNA viruses which has been demonstrated in studies with transgenic mice (174-176). Human MxA is shown to inhibit multiplication of several ssRNA and dsRNA viruses including influenza, vesicular stomatitis virus (VSV) (177), measles virus (178), Thogoto virus (179), Bunyavirus (180), and Semliki forest virus (181). MxA has also been shown to confer resistance to some DNA viral genomes (198) (164). In general, MxA appears to interfere with steps involving viral transcription although the exact mechanism of action is not known or fully understood.

MxA is a highly conserved large GTPase with additional homology to the dynamin super-family and have been found in all vertebrate species investigated so far, including mammals, birds, and fish (158). These proteins present a highly conserved N-terminal GTPase domain of ~300 amino acids, a central interactive domain of ~150 amino acids, and a GTPase effector domain (GED) of ~100 amino acids which encompasses two leucine zippers that have the capacity to form α -helices. The MxA GTPase domain has a low affinity for GTP but high GTP turnover rate which is indicative of efficient cycling achieved only within dense protein complexes typically assembled on the membrane surface (172, 173, 200). Studies with human MxA protein have shown that the GED effector domain is able to specifically contact the middle domain, and that this interaction is critical to constitute a functional GTPase domain, as well as for oligomerization (134, 189) and antiviral functions (182, 183).

The MxA gene exhibits over 590 polymorphisms (NCBI dbSNP). It was previously reported that a SNP at nucleotide position -88 (G or T) in the promoter region of the gene modulates MxA function at multiple levels including expression. MxA

mRNA is significantly up-regulated with the mutant “T” (TT or GT) allele as compared to the wild type homozygous “G” allele (201-203).

This functional polymorphism (rs2071430) is well studied and demonstrates an association with various diseases such as susceptibility to hepatitis C virus (204), SARS (205), and sub-acute sclerosing panencephalitis (206). Interestingly, an interferon stimulated response element (ISRE) is located around the single nucleotide polymorphism (rs2071430) in the promoter region and can also influence MxA promoter activity. The T allele restores and the G allele attenuates ISRE binding due to sequence homology (204, 207). Functional promoter studies using MxA luciferase reporter assay has also demonstrated that promoter activity is highest in individuals with TT genotype and lowest in the GG genotype (203, 206).

The role of MxA in prostate cancer is not fully understood. A previous study (191) implicated MxA as a potential metastasis suppressing gene in prostate cancer suggesting that its expression could be a key indicator of metastatic potential. We hypothesized that the functional polymorphism rs2071430 leading to attenuated MxA expression could be a genetic risk factor for prostate cancer. Our results demonstrate that rs2071430 polymorphism is associated with prostate cancer and that a specific rs2071430 genotype (GG) may confer an increased susceptibility to prostate cancer.

3.3 MATERIALS AND METHODS

3.3.1 Samples

The genotyping protocol and use of human samples in the study were approved by IRB at Clark Atlanta University. Genotyping was performed on a total of 304 samples.

The numbers of cancer and normal samples were approximately 50% each of the total sample set (Table I). Retrospectively collected buffy coat samples were obtained from Bio-specimen Shared Resource, KU Cancer Center, University of Kansas Medical Centre and Cooperative Human Tissue Network (CHTN, Southern Division) following appropriate protocol review and approval. The purified genomic DNA samples were obtained commercially from BioServe Inc. (Beltsville, MD). All samples were stored at -80 until analysis. De-identified comprehensive clinical information regarding age, ethnicity and stage was available for all samples. However, family history of prostate cancer, PSA level and Gleason score was not available for all samples hence were not included in final statistical analysis.

3.3.2 DNA isolation

Genomic DNA was isolated (and stored at -80C) from cultured cells or buffy coat using AquaPure total genomic DNA isolation kit (Bio-Rad). On an average approximately 30 µg of DNA was routinely isolated from 300 µl of buffy coat.

3.3.3 Genomic PCR

Genomic PCR was carried out in a 25 µl PCR reaction that consisted of 12.5ul GoTaq Colorless Master Mix (Promega), 30 ng genomic DNA and 400 pM of 5' and 3' primer each. The PCR was carried out for 35 cycles with annealing temperatures 52.9C for rs2071430. The following primers were used rs2071430: Forward 5'-TGT ATA CCT GCA AGT CAC AGG, Reverse 5'-TGT TAG TTA CTA GCA GCC GAG, nested primer for rs2071430: 5'-GAG CAC CTT GAT CCT CAG AC.

3.3.4 SNP detection

The rs2071430 polymorphism was detected by sequencing the PCR amplicon spanning the above stated SNP using the primer pairs indicated above. An aliquot of the respective PCR reaction was first analyzed on 1.5% agarose gel to confirm specificity and quality of the reaction in terms of band size and absence of any background PCR product. Once confirmed, the remaining PCR product was cleaned using ExoSAP-IT (USB) before sequencing on the AB sequencer (DNA sequencing Lab, Morehouse School of Medicine, Atlanta, GA). The sequencing was performed using a nested primer within the rs2071430 PCR product. The sequences were scanned using ABI sequence scanner and the SNPs were manually detected. The amplicons showing low quality reads were re-sequenced. The single nucleotide polymorphisms were also detected by SNPdetector (208) to ensure that the SNPs were due to heterozygous allelic variations and not due to sequencing artifacts.

3.3.5 Statistical analysis

Each of the polymorphisms was tested for association with prostate cancer. Odds ratio and 95% confidence intervals were calculated for the genotype in association with prostate cancer using logistic regression analysis with adjustment for age. Relationship between genotype distribution with subjects stratified with race and age were also analyzed using the above model. NPSS version 2007, version 07.1.19 and SigmaStat v3.5 was used for statistical analysis. The sample size calculations and power were calculated using the "Power for Genetic Association Analyses" (PGA) package in Matlab (available on the National Cancer Institute website) (209) using following parameters:

90% power, $\alpha=0.05$, cancer prevalence of 250/100,000 men (actual for all races, white and black is 156.9, 150.4 and 234.6 per 100,000 men respectively as per SEER), minor and major allele frequencies shown in table III (excluding TT allele), case control ratio of 1 and relative risk of prostate cancer in African American men as 2.5 as compared to Caucasians. Using these calculations the sample size was approximately 110 samples (± 23). This sample size was achieved in this study.

3.4 RESULTS

3.4.1 Sample demographics

A case-control study was performed to investigate rs2071430 genotype on 304 samples collected retrospectively. The samples were distributed equally ($p=0.73$) between cases ($N=164$, 53.9%) and controls ($N=140$, 46.1%). The mean age of cancer samples was 63.7 ± 0.746 years, and the mean age of normal samples was 60.2 ± 1.3 years. Both these groups were considered age matched ($p = 0.273$) (Table I). Comprehensive statistical analysis based on χ^2 analysis indicated the lack of any bias in the incidence of prostate cancer among the racial and age groups (Caucasians (53.3%) and African Americans sample sets (46.7%, $\chi^2=0.61$).

3.4.2 MxA SNP rs2071430 in population

We first wanted to understand the population distribution of the rs2071430 genotype in the normal population published in NCBI dbSNP database. The results showed that Caucasian and Hispanic subjects lack the homozygous TT genotype (Table II). The TT genotype was observed at low frequency in African Americans (4.2%) whereas in the subjects with Pacific Rim heritage, the TT genotype frequency was

highest at 16.7%. These results clearly suggested a strong racial distribution of the minor TT genotype (Table II). In the normal population, the GG genotype in NCBI database was 70% in African American (n=24) but 50% in our dataset (n=60). A recent study (210) showed that the MxA GG genotype distribution in African population (consisting of subjects from Libya, Cameroun, Niger or Rwanda, none from African American background) was 80%. The genotype distribution in our data set and those reported elsewhere (e.g. Table II) could be due to sample size and ethnic background (210).

3.4.3 Sample set frequency of rs2071430 polymorphism

The frequency of rs2071430 genotype in our normal mixed race sample set (58.6% (GG), 35.7% (GT) and 5.7% (TT)) was nearly consistent with those reported for other heterogeneous control populations (NCBI dbSNP database, Table II, PI dataset: 72.5% (GG), 22.5% (GT) and 5% (TT)). In our study, the rs2071430 allele frequency also conformed to Hardy-Weinberg equilibrium in the African American population (chi-square 2.23, df=2, $p=0.11$). There was a marked deviation from Hardy-Weinberg equilibrium in Caucasian sample set due to lack of TT genotype. The frequency distribution of rs2071430 in our complete sample set and samples stratified by race are listed in Tables III and IV respectively.

3.4.5 rs2071430 distribution in the sample and its association with prostate cancer

Each one of the genotypes was tested for its association with prostate cancer for all samples and in samples stratified by race. The combined cancer and control groups revealed that GG was a major (dominant) genotype (65.8%), whereas TT was a minor genotype (3.9%, Table III). The heterozygous genotype GT (frequency: 30.3%) was used

as a reference to calculate the association of each genotype with prostate cancer. The odds of having cancer with GG genotype was found to be 71% higher (CI=1.041 – 2.818) than the odds with GT (reference) heterozygous genotype. On the other hand, the odds of the homozygous TT genotype decreased the risk of prostate cancer by 40% but was not statistically significant (CI= 0.167-2.116). Alternatively, the GG genotype was over represented in the cancer case group (72%) as compared to the control group (58.6%) while the TT genotype was overrepresented in the control group (5.7%) compared with the cancer group (2.4%, OR=0.6, Table III).

3.4.5 Association of rs2071430 with prostate cancer and race

Sample stratification demonstrated that rs2071430 genotype distribution is associated with race. The MxA genotype frequencies in the Caucasian subpopulation were: 72 % (GG), 27 % (GT) and 1.2 % (TT). The corresponding MxA genotype frequencies for the African American subpopulation were: 58.6 % (GG), 34 % (GT) and 7.1% (TT). The genotype frequencies in both these racial sub-populations tested showed statically significant difference (chi-square 14.3, $p=0.0021$). The results clearly demonstrated that GG genotype is over-represented in the Caucasian population whereas the heterozygous GT and homozygous TT genotype is over-represented in the African American population. These observation prompted us investigate the association of each genotype with cancer in African-American and Caucasian sub-populations. Although, GG genotype was associated with prostate cancer overall and over-represented in Caucasians as stated above, no significant race specific association was observed with cancer (OR 1.37, CI 0.769 – 2.442).

The distribution of GT genotype (used as reference to calculate overall risk, Table III) displayed strong association with African American as compared to Caucasians (OR 2.07, CI 0.897 – 4.780). The GT genotype was not associated with increased risk of cancer in African American (cancer 28.2% vs. normal 24%). Surprisingly, the GT genotype showed strong association with Normal Caucasians (30.4%) as compared to cancer Caucasians (17.4%). The odds ratio of cancer incidence in the Caucasian group with GT genotype was 3.1 ($p=0.01$) implying that the odds for cancer case is lower in Caucasians with a GT genotype. Unlike the African American subset, the TT genotype was absent in the Caucasian normal group but was observed in the cancer group in both racial sub-groups (16.6%). However, based on low frequency, the TT genotype showed no statistical significance in terms of its association with the disease and between racial sub-groups. No significant association with stage, Gleason grade and MxA rs2071430 was observed (data not shown).

3.5 DISCUSSION

Together with other interferon inducible antiviral genes such as oligoadenylate synthetase 1 (OAS1) and PKR, MxA (66) polymorphisms were also shown to be associated with specific disease, for example, multiple sclerosis and viral infections such as influenza and SARS. In mice, type I IFN is effective against influenza viruses only if the IFN-induced resistance factor Mx1 is present (63).

Our previous study also demonstrated a strong association of an OAS1 polymorphism with prostate cancer with racial disparities (64). The OAS1 and prostate cancer association study prompted to ask the question whether polymorphisms in other

classical IFN inducible antiviral genes such as MxA are also common risk factors in prostate cancer. For this study we selected a single functional polymorphic marker at position -88 (rs2071430) in the promoter of MxA gene. In general the rs2071430 GG genotype promotes disease susceptibility whereas TT genotype confers protection against Hepatitis C and B virus infection (203). At the mechanistic level, the functional rs2071430 GG, GT and TT genotype results in the lowest, intermediate and highest MxA expression activity, respectively (202, 211). In *in vitro* luciferase based reporter assays and in PBMC the allele TT/GT at -88 demonstrated higher reporter activity and MxA expression in response to IFN-alpha than the GG allele suggesting a functional ISRE around rs2071430 (202). Consistent with these observations, the rs2071430 genotype was proposed to predict responsiveness to IFN-alpha therapy and susceptibility of Multiple sclerosis and Hepatitis C and B (201, 212).

There is a general lack of studies associating MxA polymorphisms with cancer. Our results suggest that the low activity, GG, genotype is a risk factor for prostate cancer whereas higher activity genotype, TT, protects against prostate cancer in general. These results are significant and suggest that low MxA expression due to GG genotype could be associated with prostate cancer especially in context of a functional study which demonstrated that MxA acts as tumor suppressor. MxA inhibits the motility and invasiveness of prostate cancer cells suggesting that its attenuated expression may promote aggressiveness of the disease (191). The GTPase activity of MxA appeared necessary and sufficient for blocking prostate cancer cell (PC3) invasiveness.

The low incidence of rs2071430 homozygous TT genotype in the Caucasian population is essentially consistent with population wide genotype distribution reported in NCBI dbSNP database (Table IV). According to previous literature, with the proximity of 35 base pairs apart the -88G>T and the -123C>A (rs17000900) SNPs have been assumed to be in linkage disequilibrium (LD) although not perfectly (213) . We also have genotyped this particular SNP collectively with rs2071430 (data not shown) but did not observe strong association with prostate cancer. These results further consolidate the association of rs2071430 with prostate cancer.

Collectively, the association studies and data stratification presented in this study suggests that rs2071430 is specifically associated with prostate cancer risk although the association between the two ethnic populations showed atypical results. Our present findings are clinically very useful for future studies to determine whether the -88G>T SNP may influence clinical outcome or is a genetic factor for increased risk or aggressiveness of prostate cancer. Furthermore, the association of two interferon inducible antiviral genes OAS1 and MxA, as shown in this study strongly suggests the possible viral etiology of prostate cancer. A larger study addressing the association between rs2071430 polymorphism and MxA expression in the prostate would be required to firmly establish whether MxA genotype is associated with altered MxA expression. Alternatively, the MxA rs2071430 genotype could lead to increased risk of prostate cancer due to complex gene-gene and/or gene-environment, (such as increased susceptibility to infections) interactions.

CHAPTER 4

FUNCTIONAL SIGNIFICANCE OF MxA IN PROSTATE CANCER

4.1 ABSTRACT

Myxovirus (influenza virus) resistance A (MxA) is an interferon regulated protein responsible for a specific antiviral state against viral infection that has been shown to be up-regulated by androgens in normal prostate epithelial cells; however, there is no known role for MxA in cancer. Recent studies have indicated that MxA has the ability to modulate apoptosis and inhibit metastasis. We analyzed different expression databases for clinical evidence to investigate the association between MxA expression and prostate cancer. GEO database (Figure 15), Oncomine (Figure 16) clinical database, and Prostate Cancer microarray study (Figure 17) searches showed that MxA expression was inversely correlated with PCa. The aim of this study was to investigate the expression MxA in human prostate cancer and its functional significance on the cancer phenotype in prostate cancer. The expression of MxA protein in cancerous prostatic tissues and normal tissues was examined by IHC. DU145 cell line was used to determine the apoptotic function of MxA in vitro. DU145 cells were stably transfected with pSM2C-MxA to knockdown MxA expression. Cell viability was determined by MTT assay. Flow cytometry and Annexin V analysis were performed to observe the apoptotic activity in DU145 cells without MxA. Expression of apoptosis-related genes was assessed by western blot.

Trans-well Migration and Soft Agar Colony Formation assays were used to determine MxA's tumorigenic potential. The expression of MxA protein was significantly decreased in cancerous prostatic tissues compared to the normal tissues. DU145 cells lacking MxA showed not only significant increase in proliferation compared with the vector controls, but also decreased apoptotic activity. The loss of MxA expression also resulted in an increase in migration in the ability to form colonies; thereby illustrating its anti-tumorigenic potential in prostate cancer.

4.2 INTRODUCTION

Mx proteins belong to a family of dynamin-like large GTPases. They play a pivotal role in the type I interferon-mediated response against a broad range of viral infections. Mx proteins are key mediators of the interferon -induced antiviral response in vertebrates and hence of great biological interest and medical importance (214). Their discovery dates back to early studies on genetically determined inborn resistance of mice to influenza viruses. The mouse Mx1 protein was originally found in influenza virus-resistant mice and was shown to have intrinsic antiviral activity.

The critical role of mouse and human Mx proteins in mediating the antiviral activity of IFNs against specific viruses became evident early on but other roles for these proteins have not been extensively studied. Results show that ovine Mx is up-regulated during early pregnancy (215), Mx proteins have vesicle trafficking abilities (187), and these proteins may be involved in mitosis (216). These studies illustrate alternative roles of Mx proteins outside of antiviral activity.

Human MxA is a 76kDa cytoplasmic protein known to be strongly induced by IFN and its expression is a preferred marker for IFN biological activity in vivo. Recent literature has shown that MxA mimics the IFN effect on motility, suggesting that it might be a critical molecular mediator of the IFN effect (191). Down-regulation of a number of IFN target genes has been reported in several studies of global gene expression in prostate cancer. Shou et al.(217) Nagano et al (218), and Schulz et al (219) showed that a significant portion of the genes whose down-regulation was associated with prostate cancer tumorigenesis or tumor progression were IFN-inducible genes, including MxA. It is of interest to determine the functional significance of MxA in prostate cancer progression.

4.3 MATERIALS AND METHODS

4.3.1 Cell culture and reagents

Cell lines and cell culture: Human prostate cancer cell lines LNCaP, DU145, and PC3 were obtained from American Type Culture Collection (ATCC, Rockville, MD). LNCaP cells were cultured in RPMI-10% Fetal Bovine Serum (FBS) with antibiotics and DU145 and PC3 cells were maintained in Hams F-12 media supplemented with 10% Calf Serum with antibiotics. Cells were cultured at 37°C in a fully humidified atmosphere containing 5% CO₂. Universal Type I Interferon was obtained from R&D Systems and used at a concentration of 300 Units/mL. A Jak Inhibitor 1 (Calbiochem) was used at concentrations of 15 nM and 30 nM for 24 hours. Docetaxel (Enzo) was used at a concentration of 1 µM/mL for 2, 4, and 24 hours.

4.3.2 Transfections

MxA was stably silenced in DU145 cells using gene specific shRNA retroviral vectors (Open Biosystems) in pSM2c vector. The cells transfected with non-silencing shRNA (RHS1707) was used as control. MxA was transiently overexpressed in LNCaP cells using A0257-MX1 (GeneCoepia). Transfections and selection of transfectants (puromycin) were performed as suggested by the manufacturer. Successful gene silencing/overexpression were confirmed by qRT-PCR and Western blot analysis.

4.3.3 RNA extraction

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) as described previously [43]. The final RNA pellet was re-suspended in diethylpyrocarbonate (DEPC)-treated H₂O at a concentration of 1 mg/mL and stored at -80°C until analysis.

4.3.4 Reverse transcription and qPCR

Total RNA was isolated from all cells using a Total RNA Extraction Kit (Omega BioTek). cDNA was generated from 4 µg of total RNA using the Superscript II cDNA synthesis kit (Invitrogen). Quantitative PCR was conducted using GoTaq qPCR reagent (Promega) and transcript levels of MxA, IFN α , IFN β , p21, p27, Cyclin D1, GAPDH, GADD45, MMP13 were measured on a Bio-Rad System. All qPCR data was normalized to GAPDH expression. Sequences of all primers are shown in Appendix Table 1.

4.3.5 Protein extraction

Protein samples were quantitated using the Bio-Rad DC Protein Assay according to the manufacturer's protocol. A standard curve was determined and sample absorbance

read at 750 nm. Samples were concentrated to 30 $\mu\text{g}/\mu\text{L}$ volume and then mixed 1:1 with 2X Sample Buffer.

4.3.6 Western blotting

Total cellular protein was prepared from cultured prostate cancer cell lines using M-PER (Thermo Scientific). Total protein was separated on 4-20% SDS-polyacrylamide gel (BioRad) and subsequently blotted onto a nitrocellulose membrane (BioRad). The blotted nitrocellulose membrane was subjected to Western blot analysis using protein specific MxA antibody (Proteintech Lab Group). After washing with 1x PBS with 0.5% Tween 20, the membranes were incubated with a secondary antibody against rabbit/mouse IgG and the signal was visualized using the Super Signal West Dura Extended Duration Substrate (Thermo Scientific) and blots visualized using the Fuji Film LAS-3000 Imager.

4.3.7 Immunohistochemistry

Prostate cancer tissue microarrays (TMA) consisting of 8 normal and 40 cancer cores were purchased from US BioMax (Rockville, MD). Tissue microarray slides were de-paraffinized in xylene and re-hydrated using standard protocols. Antigens were retrieved by autoclaving in 0.01 M sodium citrate buffer pH 6.0 at 121°C/20 psi for 30 min. The slides were then blocked for peroxidase activity in 3% H_2O_2 (in PBST: PBS with 0.05% Tween 20) for 10 min and then blocked in 10% goat serum (PBST with 1% BSA) for 2 h at room temperature. The blocked sections were incubated overnight at 4°C with primary antibody (1% BSA in PBST). The slides were then washed twice with PBST for 5 min each, and then incubated with secondary antibody (1% BSA in PBST,

1:1000, HRP- goat anti-mouse; Thermo Scientific, Rockford, IL) for 1 h. The slides were washed with PBST for 5 min and stained with DAB for 2 min. Slides were then counterstained in hematoxylin and mounted with Immuno-mount (Thermo Scientific), examined and photo-micrographs taken using the Zeiss fluorescent microscope with an AxioimCam version 4.5 imaging system.

4.3.8 MTT proliferation assay

DU145+NS and DU145-MxA cells were used as target cells for the detection of any differences in the bioactivity in the absence of MxA using CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega). Cells were seeded (5×10^3 cells/well) in 96-well plates in recommended growth media. After allowing cells to attach overnight, medium was again replaced and 15 μ l Dye Solution added and incubated at 37 °C in the dark for 4 h. Following 4 h incubation, solubilization/stop solution was added and incubated for 1 h. Absorbance was read at 570 nm using a 96-well plate reader. Three independent experiments were carried out with similar results.

4.3.9 Flow cytometric analysis of cell cycle and apoptosis

The LNCaP and DU145 transfected cells were cultured in 24 well plates to a sub-confluent density. After culture, the cells were collected by trypsinization and washed with phosphate buffered saline. The cells were then fixed with 70% ethanol and stored at -20°C overnight. The following day, cells were washed twice with ice cold phosphate buffered saline PBS/FCS (10%) followed by a final wash in 1 \times PBS. The cells were then finally resuspended 1 mL of PBS (1 \times) containing 50 μ g/ml RNase A, 0.1% TritonX-100 and 1 mM EDTA and then incubated at 37°C for 30 minutes. Finally, 20 μ g/mL of

propidium iodide was added. Data acquisition and analysis were performed on an Accuri CFlow flow cytometer (CCRTD, Clark Atlanta University, Atlanta, GA). The cell cycle profiles were then analyzed using FLOWJO (for cell cycle analysis) and CFlow for apoptosis. At least 50,000 cells in each sample were analyzed to obtain a measurable signal. All measurements were performed using the same instrument settings.

4.3.10 Scratch wound assay

The migratory properties of DU145 transfected cells were measured using a scratch wound assay. Cells were plated in 6-well plates (5×10^5 cells/well) in Ham's F-12 with 5 % FBS and cultured overnight. Wells were scratched down the middle with a 200 μ l pipette tip. Culture media were replaced with Ham's F-12 containing 5 % FBS. Cells were allowed to migrate across the scratch for 48 h. Images of the scratch area were recorded at 0, 24 and 48 hours, respectively.

4.3.11 Migration assay

In vitro cell migration assay was performed using 24-well trans-well inserts (8 μ m; ref. 26). Briefly, cells were washed once with F-12 and harvested from cell culture dishes by EDTA-trypsin into 50 ml conical tubes. The cells were centrifuged at $500 \times g$ for 10 minutes at room temperature; pellets were re-suspended into media supplemented with 0.2% bovine serum albumin at a cell density of 3×10^5 cells/ml. The outside of the trans-well insert membrane was coated with 50 μ l of rat tail collagen (50 μ g/ml) overnight at 4°C. The next day, aliquots of rat tail collagen (50 μ l) were added into the trans-well inserts to coat the inside of the membranes. The inserts were left to stand for 1.5 h at room temperature before being washed thoroughly with 3 mL of F-12. Aliquots

of 100 μ L cell suspension were loaded into trans-well inserts that were subsequently placed into the 24-well plate. The trans-well insert-loaded plate was placed in a cell culture incubator for 5 h. At the end of the incubation, trans-well inserts were removed from the plate individually; the cells inside trans-well inserts were removed by cotton swabs. The cleaned inserts were fixed in 300 μ L of 4% paraformaldehyde (pH 7.5) for 20 minutes at room temperature. Cells were stained using HEMA 3 staining kit (Fisher Scientific, Inc.). Stained cells were counted in four non-overlapping low-power fields of a light microscope, and the average number of cells reflected the cell migration status in each trans-well insert. To avoid experimental bias, a systematic random sampling technique was applied in the selection of representative fields, in which sample preparation and handling was executed by different persons. Results were expressed as migration index, which is defined as the average number of cells per field for test substance / the average number of cells per field for the medium control. Each experiment was repeated at least three times using a different cell preparation.

4.3.12 Colony formation in soft agar

Cells were incubated in the presence of 0.5 μ g/mL Puromycin before reseeding in soft agar at low density to ensure the selection of transfected cells. After trypsinization and counting, the cells were resuspended in medium containing 0.3% agarose and 0.5 ml containing 500 cells was added to each well of 6-well plates. The cells were incubated at 37°C in a humidified incubator with 5% CO₂ in for 14-20 days and colonies were counted in an inverted phase contrast microscope.

4.4 RESULTS

4.4.1 Prostate cancer tissues express less MxA

We first analyzed the expression of MxA by IHC staining in a collection of prostate cancer and normal tissue microarray slides. As shown in Figure 18, the MxA protein was virtually undetectable in the highest grade of prostate cancer but highly expressed in normal prostatic tissue. The cellular localization of MxA was mostly cytoplasmic with some perinuclear staining as indicated by the arrows. These results indicated that MxA has lower expression in clinical prostate cancers, suggesting MxA as a promising target for prostate cancer therapy. This prompted us to investigate the expression pattern of MxA in our prostate cancer cell lines.

4.4.2 Expression of MxA in prostate cancer cells

It is known that MxA is an interferon inducible protein so the expression of interferon alpha and beta were examined in the prostate cancer cell lines to see if the level of interferons present correlates to the expression of MxA in the prostate cancer cell lines (Figure 19). The expression of MxA was variable in all cells as shown in Figure 20. The normal prostate cell line RWPE1 exhibited the highest expression (data not shown) which correlates well with our tissue microarray data.

4.4.3 Regulation of MxA

In addition to MxA being IFN inducible we wanted to investigate the alternative mechanisms by which MxA is regulated. We are reporting for the first time that MxA is also a serum regulated gene in prostate cancer cells. Cells cultured in calf serum exhibit constitutive expression of MxA, while cells cultured in FBS have reduced expression of

the gene as shown in Figure 21. In androgen sensitive LNCaP cells; androgens down-regulate the basal expression of MxA (Figure 22). In cells which constitutively express MxA, they are sensitive to the interferon pathway as illustrated in Figure 23.

We also wanted to investigate the possible viral dependent mechanism of regulation. We used the prostate cancer cell line 22Rv1. These cells illustrated no expression of endogenous interferons but exhibited higher expression of MxA. It is also known that 22Rv1 does produce high titer of the XMRV virus. We assayed all cell lines for XMRV as shown in Figure 24. 22Rv1 cells were the only population to contain the virus while all other prostate cancer cell lines were negative. These results confirm viral dependent regulation of MxA.

4.4.4 MxA overexpression leads to cell cycle arrest

In this study we wanted to investigate the functional significance of MxA in prostate cancer cells. We attempted stable transfection of MxA in LNCaP cells which proved to be lethal. Consistent with previous literature, permanent high-level synthesis of MxA is detrimental (199); however, transient over-expression of MxA showed a significant G1 cell cycle arrest as seen in Figure 25. The G1 checkpoint is where eukaryotes typically arrest the cell cycle if environmental conditions make cell division impossible. This arrest was apparent by a significant up-regulation of p21 and down-regulation of Cyclin D1 (Figure 26).

4.4.5 Silencing of MxA increased proliferation of DU145 cells

Because of the complexities of overexpression of MxA, DU145 model system was used to silence MxA because of its constitutive expression as illustrated in Figure 27.

To explore the effect of MxA silencing on cell growth, MTT assays were performed at 24, 48, and 72 hours, respectively, after stable transfection. We wanted to analyze the effects of silencing MxA on the growth rate of the cells. Results showed that there was an increase in cell proliferation in MxA silenced DU145 cells as compared to the Non-specific control (Figure 28). To further explore the effect of MxA knockdown, cell cycle analysis was performed in Figure 29. These cells were rapidly progressing through the cell cycle which supports our data of increased proliferation.

4.4.6 MxA is a tumor suppressor in prostate cancer

After seeing the effects of loss of MxA on proliferation we also wanted to see what other effects it may have on the cancer phenotype. A scratch wound assay showed that knockdown of MxA caused the wound in DU145-MxA cells to heal faster than the non-silenced control cells in Figure 30. These results were confirmed in a Trans-well migration assay illustrated in Figure 31. Cells with constitutive MxA were less migratory than the transfected cells. There was also an increase in MMP-13 in MxA knockdown cells (Figure 32). Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes as well as in disease processes, such as metastasis. This may provide some evidence as to the mechanism by which motility increases in these cells.

Our results show a strong evidence of MxA as a tumor suppressor in prostate cancer. To confirm our hypothesis we performed a soft agar colony formation assay in Figure 33. Anchorage-independent growth is one of the hallmarks of cell transformation, which is considered the most accurate and stringent in vitro assay for detecting malignant

transformation of cells and/or mechanism that block transformation phenotype. The soft agar assay is the closest *in vitro* assay to *in vivo* studies. The results in Figure 34 confirmed that cells with silenced MxA has greater colony forming units (CFU) which illustrates an anti-tumorigenic role for MxA.

4.4.7 MxA sensitizes prostate cancer cells to chemotherapeutic drugs

DU145 control and DU145-MxA cells were exposed to the chemotherapeutic drug Docetaxel (DTX) for a period of 24 hours and Annexin V staining was performed. Cells with MxA were more sensitive to DTX induced apoptosis as shown in Figure 35. This suggests a possible role of MxA as a novel prostate cancer therapeutic target. To further investigate this we subjected the cells to immunocytochemistry with DTX treatment in DU145 cell that express MxA. MxA did co-localize with alpha-tubulin before treatment with DTX and after treatment that localization was disrupted as depicted in Figure 36. MxA had very diffuse cytoplasmic staining before treatment; however, after treatment MxA was localized in punctate dots. This intriguing observation will be investigated further but may provide some mechanism of how MxA sensitizes these cells to chemotherapeutic agents.

4.5 DISCUSSION

Our studies have for the first time shown an anti-tumor role for MxA in prostate cancer. This study began with the database analysis identifying MxA expression as inversely correlated with prostate cancer, and the notion that MxA has alternative functions as antiviral activity. As demonstrated here through prostate cancer tissue microarray a significant difference was seen in MxA protein expression. There is an

abundance of MxA in normal tissue but continuously decreasing amounts of MxA the more malignant the cancer, consistent with current literature (219). The prostate cancer cell lines demonstrated variable expression of MxA but overall expression was low. The overall low expression of MxA in prostate cancer could possibly be due to the polymorphism in MxA's proximal promoter region. Our previous study established an association of MxA with prostate cancer risk, specifically the genotype with low promoter activity (220) which results in reduced expression of MxA.

In the prostate cancer cell line, LNCaP, the basal expression of MxA is down-regulated by androgens. This phenomenon was supported by the results that MxA is also serum regulated. Cells that were cultured in fetal bovine serum (FBS) had no expression of MxA but cells cultured in calf serum (CS) exhibited constitutive expression of MxA. Previous work using immortalized human meibomian gland and conjunctival epithelial cells, androgens down-regulate genes involved in processes such as the immune response and JAK-STAT signaling cascade (221). The level of androgens present in FBS alone seems to be sufficient to decrease expression of MxA including treatment with exogenous androgens. Cells with constitutive expression of MxA are sensitive to the classical interferon signaling pathway, but 22Rv1 cells also illustrate viral-dependent regulation of MxA. This area of viral dependent regulation of MxA in prostate cancer needs to be further investigated.

To elucidate the functional significance of MxA in prostate cancer and its effect on the cancer phenotype we first looked at ectopically expressing MxA in LNCaP cells. Expression of exogenous MxA in LNCaP cells resulted in significant G1 cell cycle arrest.

This was supported by a reduction in Cyclin D1 and an up-regulation of p21. It is known that degradation of Cyclin D1 is sufficient to cause a G1 cell cycle arrest (222, 223). We believe this down-regulation of Cyclin D1 to result from MxA's up regulation of p21. p21 can down-regulate Cyclin D1 in a p53 independent or dependent manner (224). We believe this to be p53 dependent due high level synthesis of MxA (199) resulting in a possible increase in p53 and apoptosis, thus there was no generation of a stable MxA expressing cell line.

Because of this we knocked down the expression of MxA cDNA in the prostate cancer cell line DU145 and compared this effect with that of a non-specific control vector. Loss of MxA resulted in a slight increase in proliferation. There was also an increase in the G2 cell cycle of cells with knock-down MxA (22.7% vs 13.9%). G2 phase is a period of rapid cell growth and protein synthesis during which the cell prepares for mitosis (225). These results are consistent with the increase in proliferation. Taken together these results prompted us to investigate MxA's effect on cell motility in prostate cancer. According to a report published by Mushinski et al., MxA inhibits motility comparable to IFN (191). IFN inhibits normal cell motility (226). Their results suggest that MxA may be a critical molecular mediator of the IFN effect. Our results corroborate the current literature to show that loss of MxA results in increased wound healing. The trans-well migration assay quantitated the findings of the scratch wound assay. Because the degradation of the extracellular matrix and the basement membrane by proteases, such as matrix metalloproteinases (MMPs) is critical for migration and invasion of cancer cells, we investigated the expression of several MMPs. The DU145-MxA cells also

showed an increase in MMP13 expression. This may provide some mechanism by which motility is increased in these cells as compared to the vector control. Increased expression of MMP13 (227) and MMP9 are known to increase migration and motility and decreases in these two MMPs have been shown to inhibit migration and invasion in oral cancer (228).

Anchorage-independent growth is one of the hallmarks of cell transformation and uncontrolled cell growth. The soft agar colony formation assay is considered the most accurate and stringent in vitro assay for detecting malignant transformation of cells by measuring proliferation in a semisolid culture media. Our results demonstrate that depletion of MxA leads to increased soft agar colony formation. This set of results in conjunction with increased proliferation and migration after reduction of MxA suggests an alternative function of MxA as an inhibitor of tumorigenesis in prostate cancer. Expression of functional tumor suppressor genes should inhibit or limit the ability of cells to grow in an anchorage independent manner (229-231) .

Consistent with our results illustrating MxA as a possible tumor suppressor in prostate cancer our next goal was to identify if MxA was able to sensitize prostate cancer cells to chemotherapeutic drugs. This was achieved by the treatment of DU145 transfected cells with DTX. Cells with reduced levels of MxA also exhibited fewer cells undergoing apoptosis. The cells with constitutive expression of MxA were more sensitive to the chemotherapeutic agent and resulted in increased apoptosis. This provides more compelling evidence for MxA's anti-tumorigenic potential. Previous studies have demonstrated that MxA interacts with tubulin (191) and other subcellular components

(232). Studies have associated microtubules in migration (233-235). The chemotherapeutic drug DTX is a clinically well-established anti-mitotic chemotherapy medication. The main mode of therapeutic action of DTX is the suppression of microtubule dynamic assembly and disassembly (236). Our data suggest that MxA may be important in this process so we wanted to see the interaction of MxA and alpha tubulin. The results indicated that MxA and alpha tubulin co-localize but the interaction is abrogated after treatment with DTX. This may provide some evidence of how MxA sensitizes the cells to chemotherapeutic drugs as well as its tumor suppressive functions because DTX may be causing MxA to localize and form punctate dots because of its role in microtubule dynamics. This area will be investigated further to determine the overall significance. The results of the present study are particularly novel and supportive to the alternative functions of MxA to include a role as a possible tumor suppressor in prostate cancer. These results could also prove to be profound in clinical translation and advocate a new strategy for novel therapeutics in prostate cancer progression.

CHAPTER 5

CONCLUSION

In summary, we have shown reduced MxA expression is correlated with prostate cancer. The results presented in this study demonstrate that MxA has anti-tumor properties. MxA plays an important role in normal prostate epithelial cells by inhibiting proliferation and inducing apoptosis. The expression of MxA is decreased in prostate cancer tissue; although MxA is expressed variably in all prostate cancer cell lines at both the transcript and protein (low) levels. The explanation behind why the expression of MxA is decreased could be attributed to its promoter polymorphism rs2071430. This polymorphism which is a key factor in expression of MxA is also a significant risk factor for prostate cancer. The wild type homozygous “GG” genotype results in low promoter activity/low expression and increased prostate cancer risk. The mutant “TT” genotype is associated with high promoter activity/higher expression which was consistent with our data. This mutant genotype was increased in normal samples than the cancer samples. MxA is the preferred marker of interferon activity and has mainly been implicated in studies related to interferon functions. MxA has been shown to function by the classical IFN pathway but we have shown this protein to be regulated by viral-dependent, XMRV, and viral-independent pathways including serum factors and androgens. Overexpression of MxA causes a G1 cell cycle arrest possibly due to down-regulation of Cyclin D1 by a

p53 dependent up-regulation of p21. Successful stable knock-down of MxA initiates increased proliferation, increased cell cycling, increased migration through the up-regulation of MMP13, and a decrease in apoptosis. MxA has been shown to sensitize prostate cancer cells to chemotherapeutic drugs perhaps through a microtubule dependent mechanism which needs further investigation. The most compelling evidence presented through this study was the increased formation of colony forming units in cells with depleted MxA. Collectively the data reveals the anti-tumorigenic role of MxA in prostate cancer.

APPENDIX

Figure 1

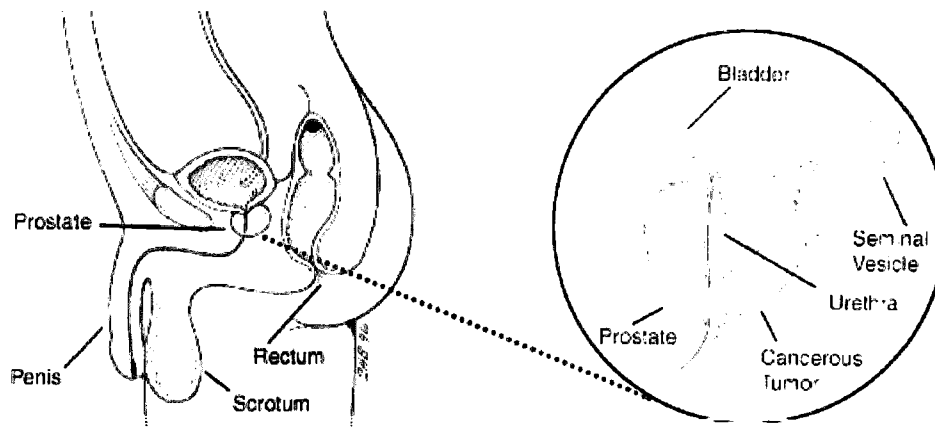


Figure 1: The prostate is a gland found only in males. It is located in front of the rectum and below the urinary bladder. The size of the prostate varies with age. In younger men, it is about the size of a walnut, but it can be much larger in older men. Figure taken from (237)

APPENDIX

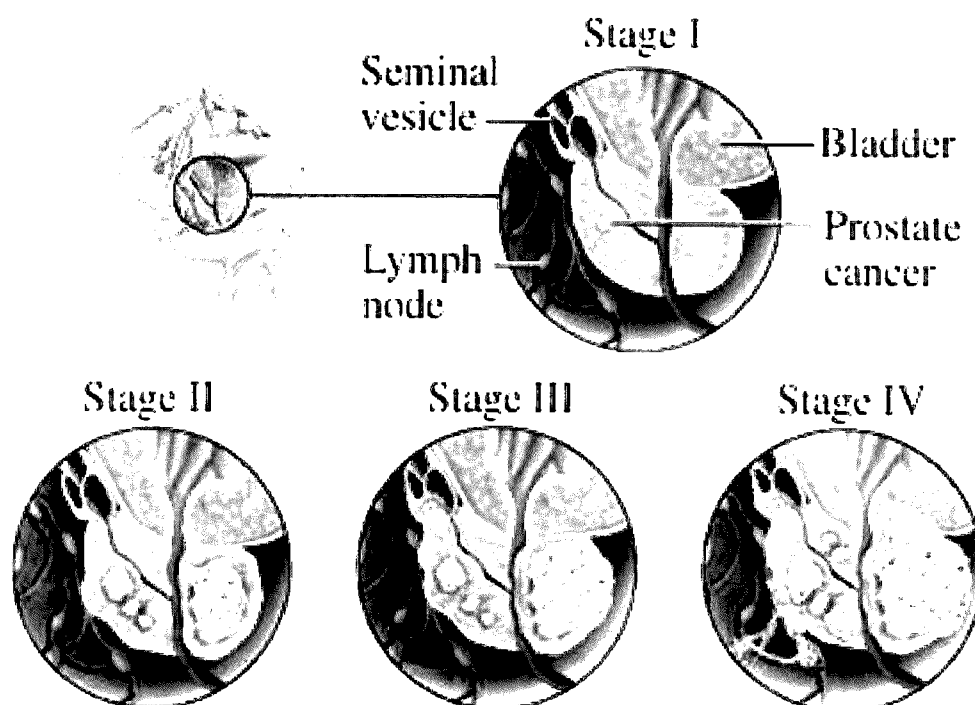
Figure 2

Figure 2: The stages of prostate cancer. In stage I, prostate cancer is confined only in the prostate gland area. In stage II, the prostate tumor has grown inside the prostate but hasn't yet extended beyond it. Stage III prostate cancer has spread outside of the prostate minimally. Prostate cancer in stage III may involve nearby tissues. In stage IV, the cancer has metastasized outside of the prostate to other tissues. Figure taken from (238)

APPENDIX

Figure 3

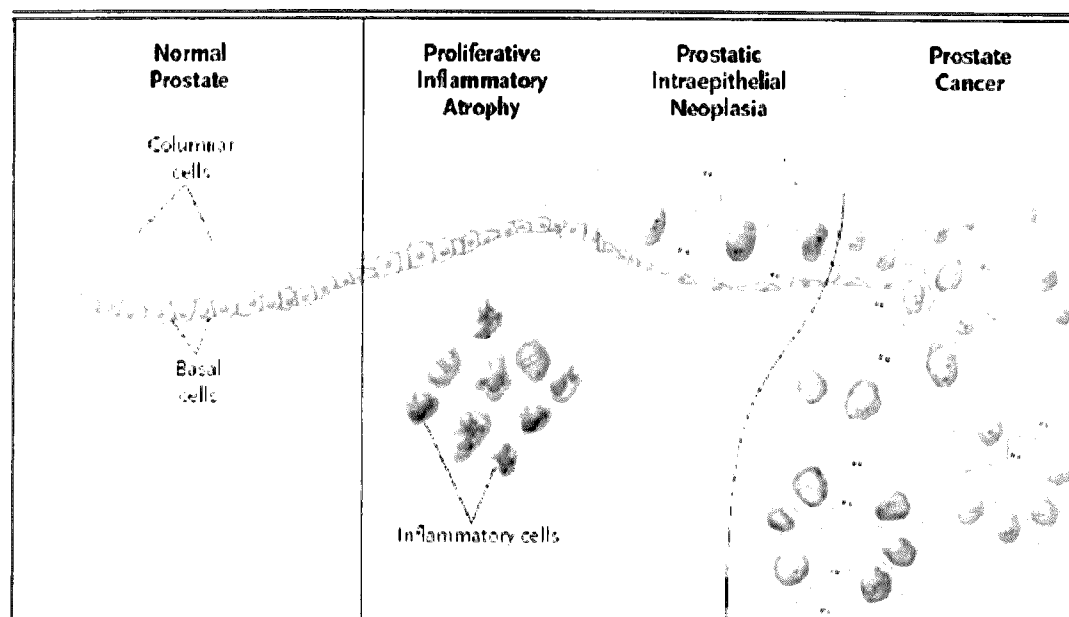


Figure 3: Proliferative Inflammatory Atrophy (PIA) to Prostatic Intraepithelial Neoplasia (PIN) and Prostate Cancer. PIA may represent an intermediate step between normal tissue and PIN and may, therefore, serve as a risk factor lesion for prostate cancer. Figure obtained from (40).

APPENDIX**Figure 4**

<u>VIRUS</u>	<u>ASSOCIATED CANCERS</u>
Hepatitis B and Hepatitis C	Hepatocellular carcinoma (liver cancer)
Epstein-Barr virus	Burkitt's lymphoma, Nasopharyngeal carcinoma, Hodgkin's lymphoma
Kaposi's sarcoma-associated herpes virus	Kaposi's sarcoma
Human papillomaviruses	Cancers of the cervix, anus, penis, mouth, and skin
Human T-lymphotropic virus	Adult T-cell leukemia

Figure 4: Viruses and associated cancer types. Several viruses have been implicated in the imitation or etiology of various cancers.

APPENDIX

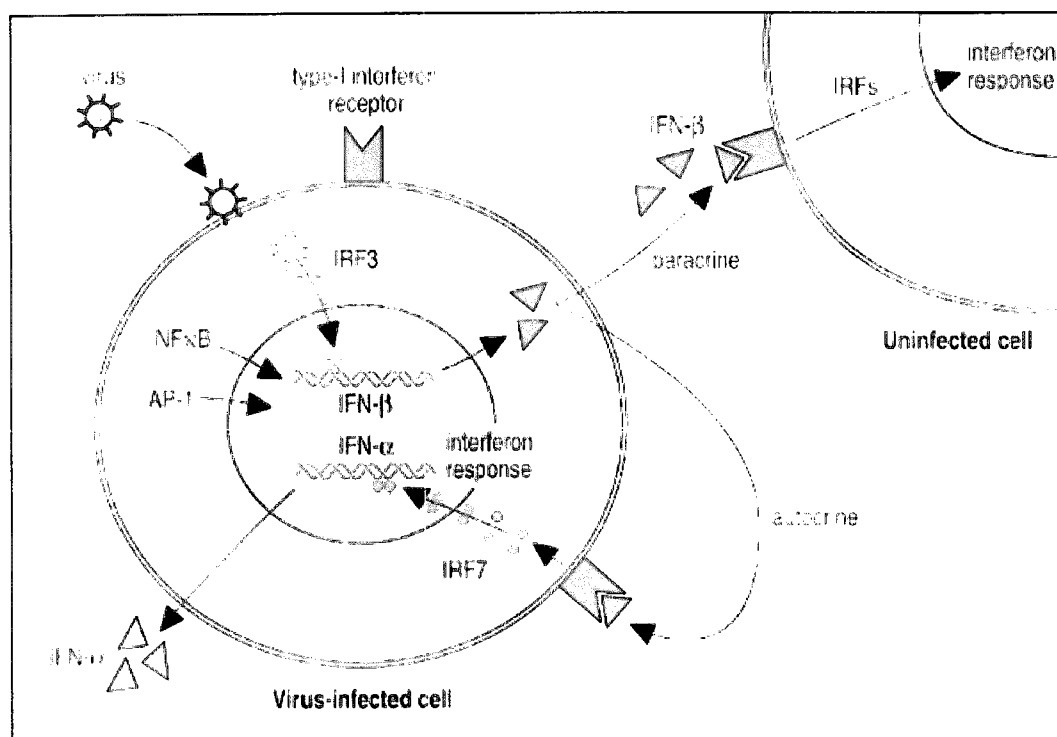
Figure 5

Figure 5: Secretion of interferon in an paracrine fashion. The cell on the left is infected with a virus that triggers signals that lead to the secretion of IFN- β which will bind to the interferon receptor expressed by nearby cells that are not infected by the virus, acting in a paracrine fashion to induce the interferon response that helps these cells to resist infection. As depicted in the lower half of the cell, secretion may also occur in an autocrine fashion. Figure obtained from (239)

APPENDIX

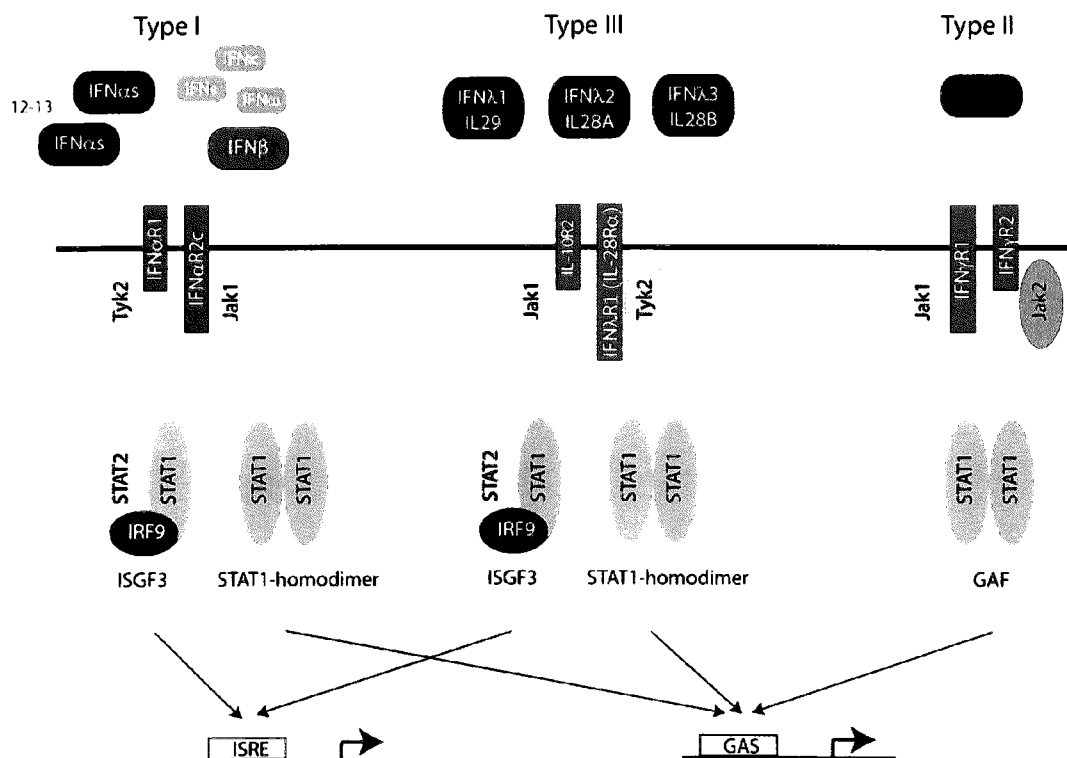
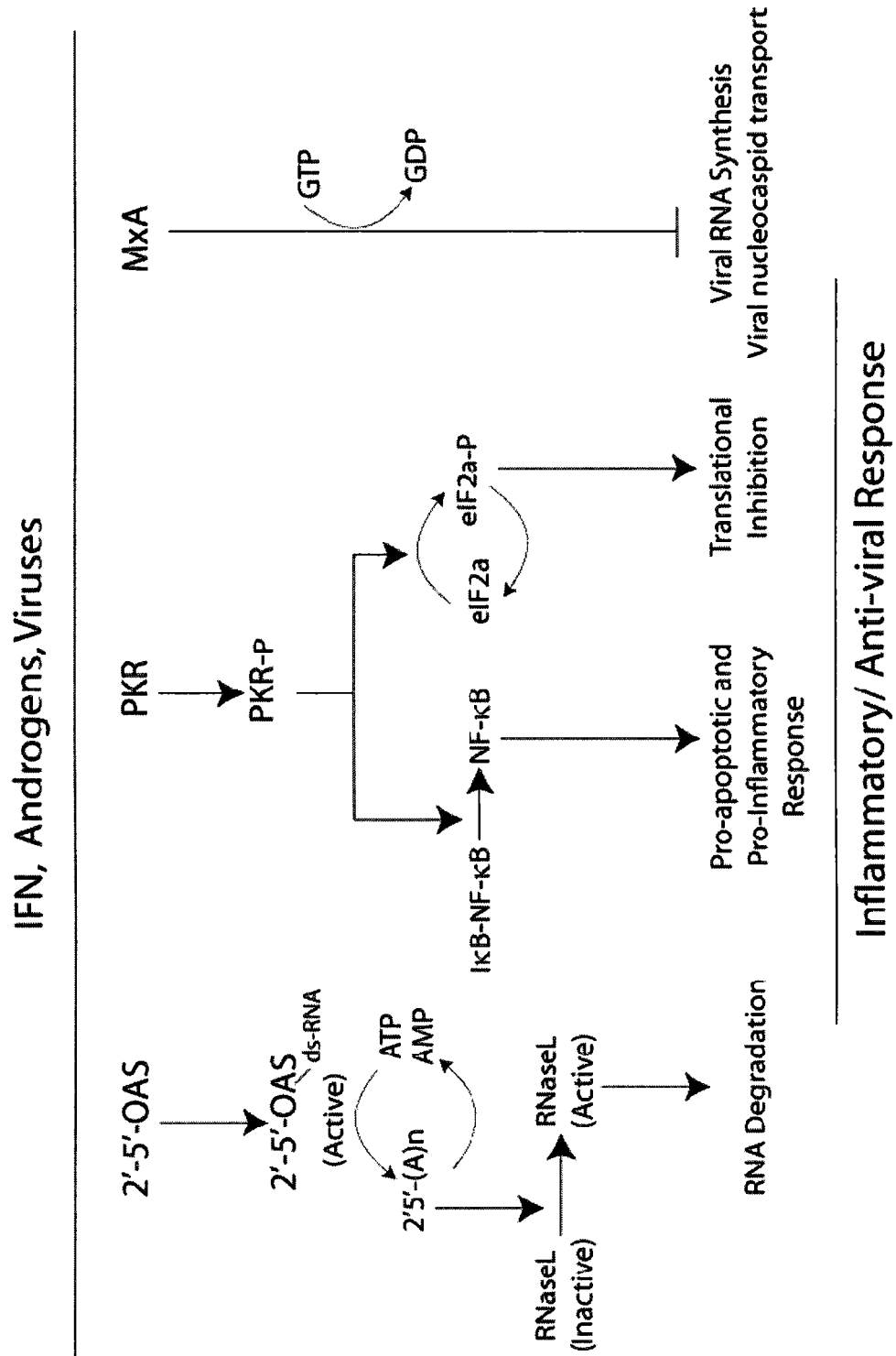
Figure 6

Figure 6: Type I and III IFNs bind to distinct receptors, but activate the same downstream signaling pathways, and induce a widely overlapping set of genes through the activation of IFN stimulated gene factor 3 (ISGF3) and STAT1 homodimers. IFN γ , the only type II IFN, activates STAT1 homodimers, but not ISGF3, and thereby induces an overlapping but distinct set of ISGs. Figure taken from (240)

APPENDIX

Figure 7



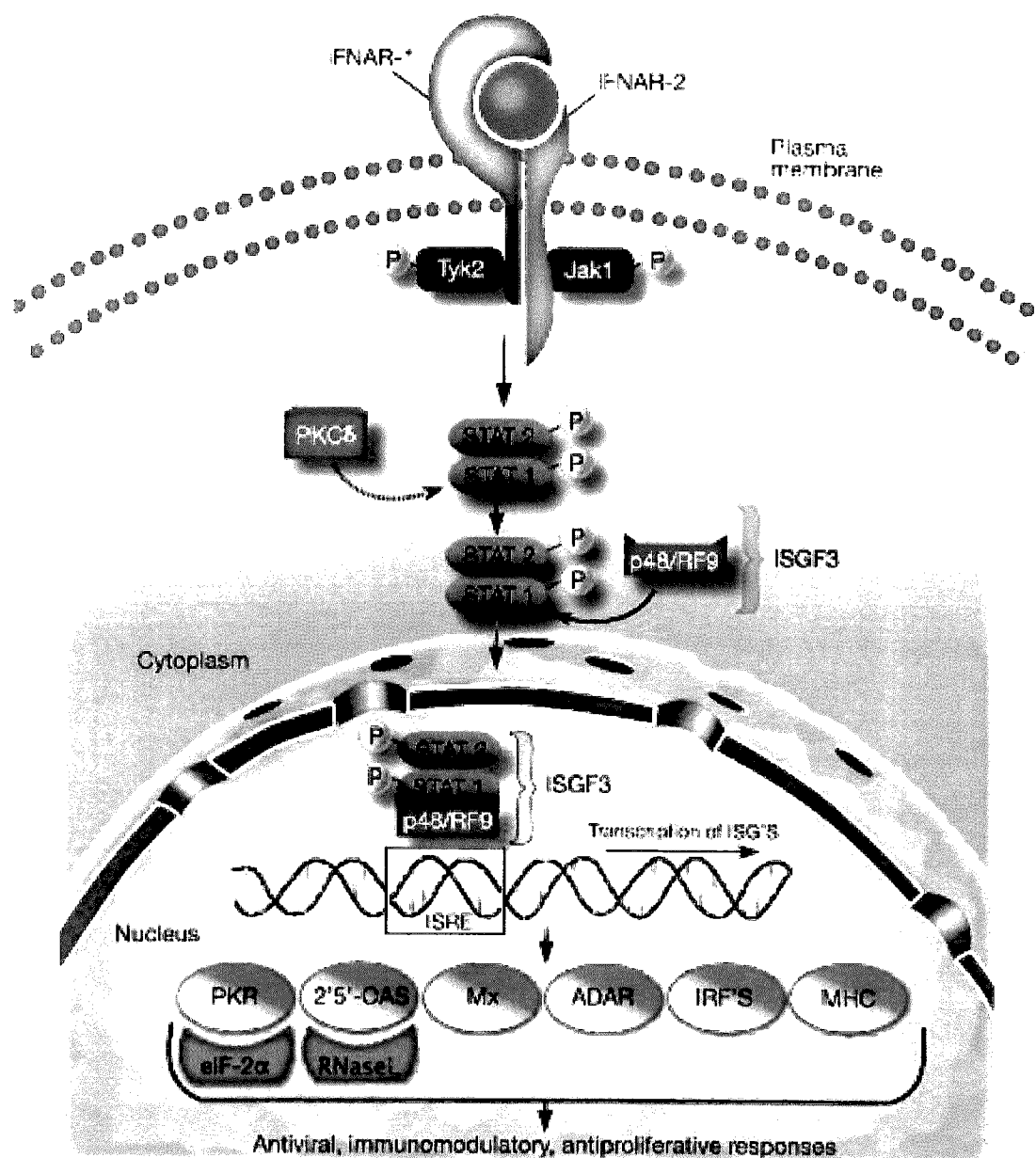
APPENDIX

Figure 7: The three major Interferon type I-induced antiviral pathways. 2. '5'

oligoadenylate synthetase (OAS) is involved in RNA degradation. The double stranded RNA (dsRNA)-dependent protein kinase (PKR) is responsible for inhibition of translation and the MxA protein pathway functions by inhibiting virus RNA synthesis and the transport of viral nucleocapsids.

APPENDIX

Figure 8

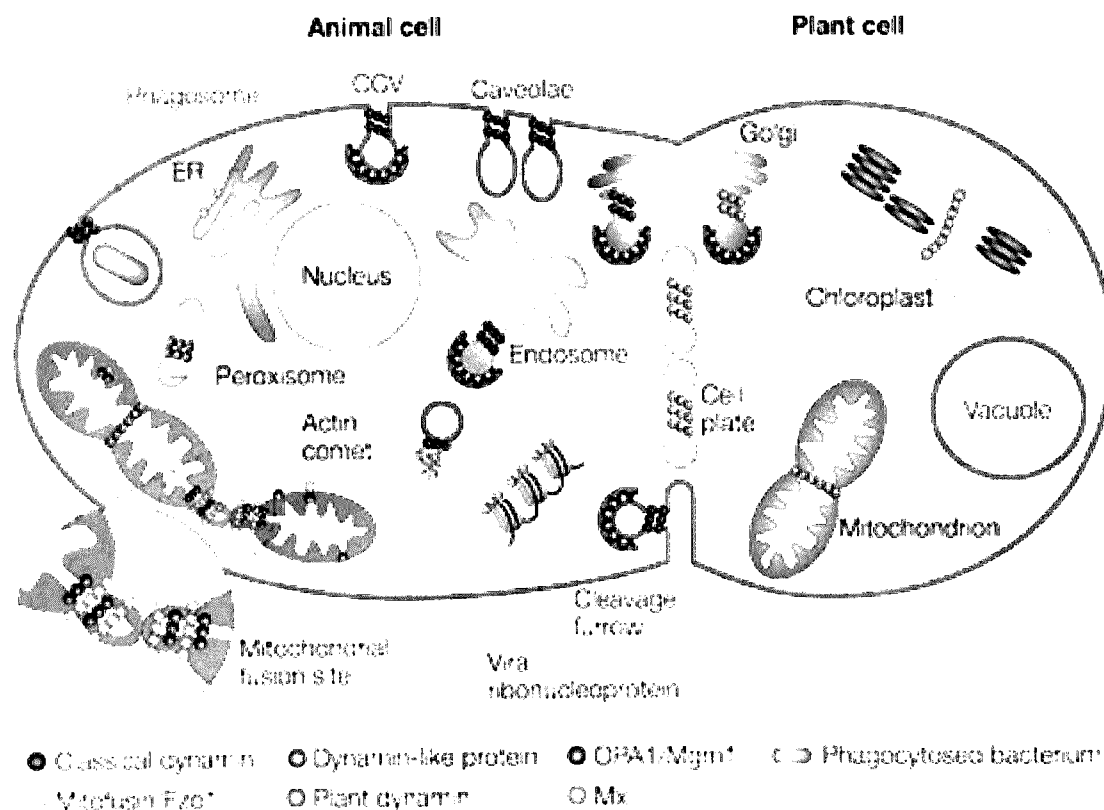


APPENDIX

Figure 8: There are numerous different type I IFNs in humans, and they all signal through the same cell surface receptor consisting of two chains called IFNAR1 and IFNAR2; yet they can have markedly distinct cellular activities. Once the ligand binds to the IFNAR1/IFNAR2 receptor complex the intracellular Jak-STAT pathway is activated. Jak1 and Tyk2 are phosphorylated and STAT1 and STAT2 are phosphorylated and heterodimerize. They then recruit ISGF3 which enters the nucleus and binds to the interferon stimulated response element to initiate transcription of interferon stimulated genes. Figure obtained from (241)

APPENDIX

Figure 9



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Figure 9: Dynamin superfamily members and their cellular localizations in animals and plants. The dynamin family is divided into classical dynamins and the dynamin-like proteins. Figure taken from (133)

APPENDIX

Figure 10

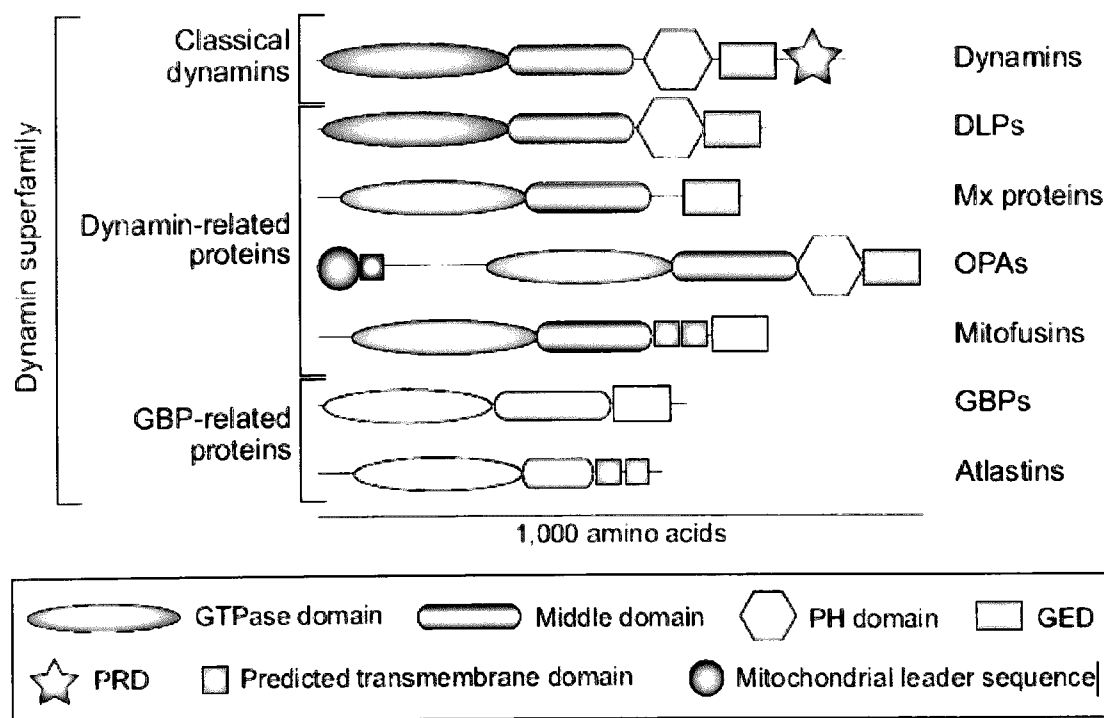
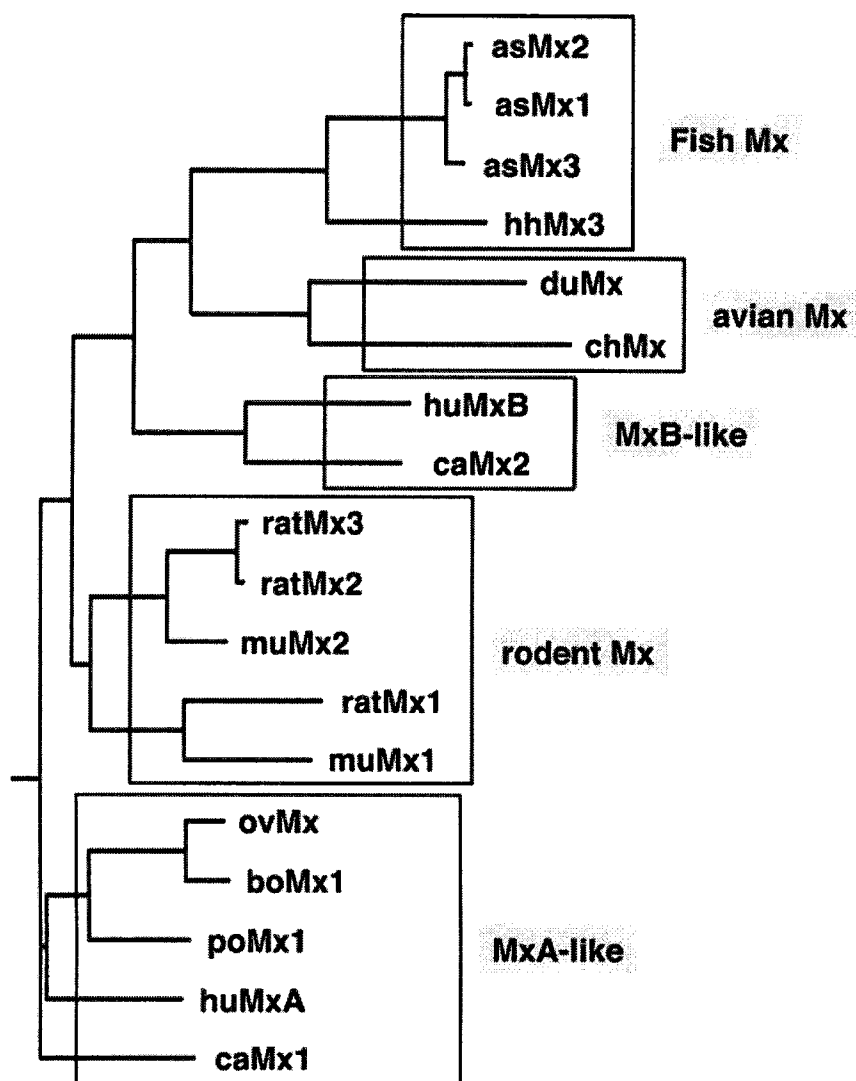


Figure 10: Domain architecture of the dynamin superfamily members. A cartoon comparison of domain organization between different family members as indicated: classical dynamins, dynamin like protein (DLPs), Mx proteins, Optic atrophy proteins (OPAs), mitofusins, guanylate-binding proteins (GBPs) and atlastins. Figure from (133)

APPENDIX

Figure 11



APPENDIX

Figure 11: Phylogenetic tree of Mx proteins. According to sequence similarities, currently known vertebrate Mx proteins can be classified into five subgroups: fish Mx, avian Mx, MxB-like, rodent Mx and MxA-like. as: Atlantic salmon; hh: Atlantic halibut; du: duck; ch: chicken; hu: human; ca: canine; rat: rat; mu: murine; ov: ovine; bo: bovine; po: porcine. Figure adopted from (242)

APPENDIX

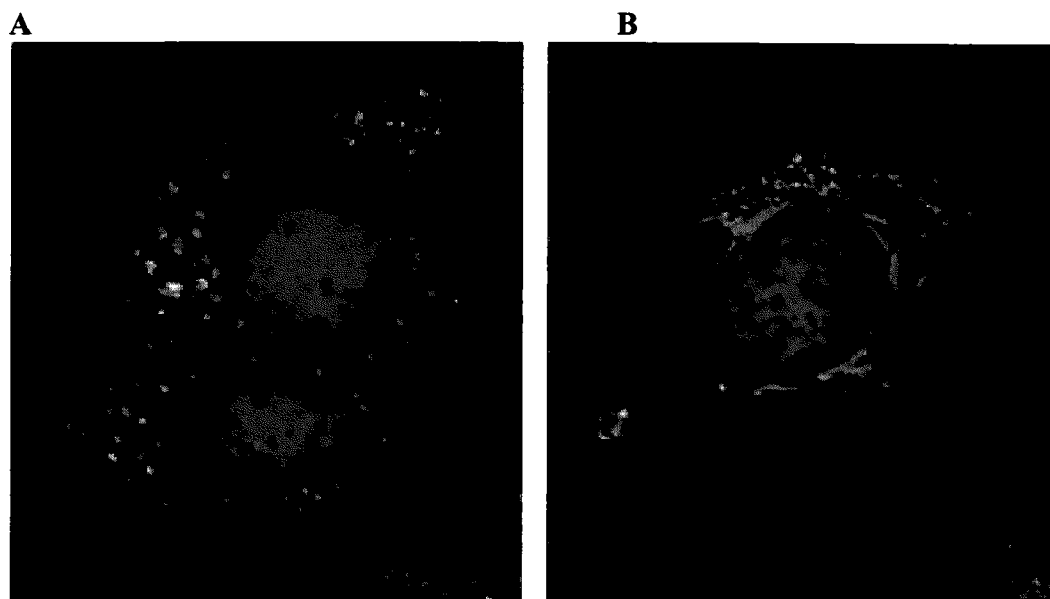
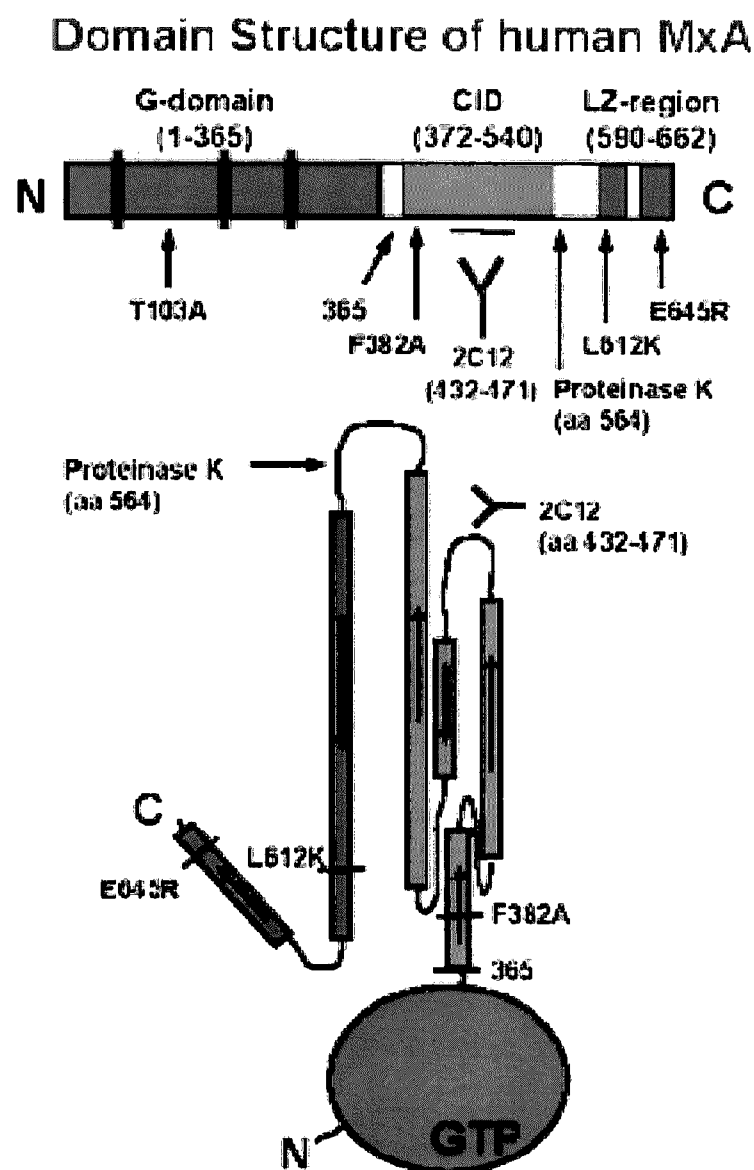
Figure 12

Figure 12: MxA antiviral activity during La Crosse virus infection. MxA GTPase interferes with the intracellular transport of essential viral components. Membrane-associated MxA blocks the transport of virus nucleocapsid protein by sequestering the viral protein into large perinuclear aggregates. A) MxA illustrated by the green dots forms small dots in uninfected cells. B) After viral infection MxA redistributes together with the viral nucleoproteins into perinuclear complexes. The cytoskeleton is stained red for microtubule and the DNA is stained in blue. Figure taken from (243)

APPENDIX

Figure 13



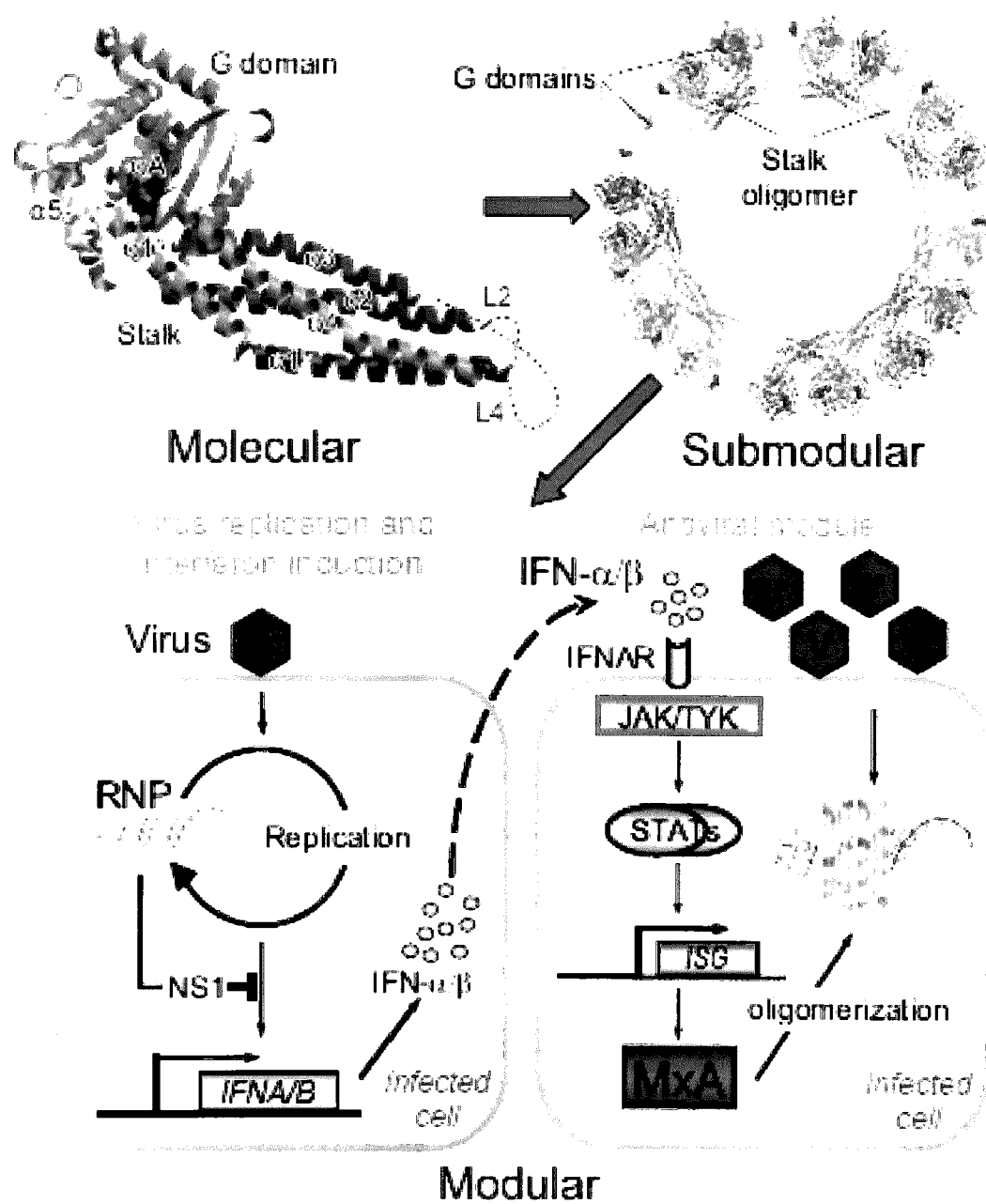
Functional domains of MxA and a model of the putative structure according to the tertiary structure determined for IrGBP-1 by Prakash et al., (2000) Nature 403, 567.

APPENDIX

Figure 13: Hypothetical domain arrangement of human MxA. The GTP-binding domain (88) contains the tripartite GTP-binding element (red bars) and the “self-assembly domain”. The C-terminal effector domain contains the “central interactive domain” (CID; orange) and a “leucine zipper” region (LZ; green), also called “GTPase effector domain” (GED) in the case of dynamin. Figure taken from (130).

APPENDIX

Figure 14



APPENDIX

Figure 14: Functional organization of the antiviral module containing hsMxA. Upper left: hsMxA monomer corresponds to a molecular component of the antiviral module. Upper right: an hsMxA oligomer model represents a submodule with a defined subfunction in the antiviral module. Lower: the proposed antiviral module comprises components needed for the induction and regulation of hsMxA activity (e.g. Type I IFNs). IFN α/β are induced by viral RNA produced during viral replication leading to activation of a cascade of intracellular receptors and transcription factors that activate the IFN promoters. The FLUAV NS1 protein is an example of a viral antagonistic protein known to suppress IFN induction. Newly synthesized and secreted IFNs bind to IFNAR and activate the expression of ISGs including hsMxA via activation of JAK/TYK and STAT transcription factors. Upon infection, cytoplasmic hsMxA might recognize the incoming viral RNP structures and self-assemble into rings resulting in a stable complex that blocks viral RNP function. Figure taken from (244).

APPENDIX

Table 1. Demographics of all samples used in the study. Table obtained from (220)

Sample type	Total no. of Samples (N)	AGE			P Value
		Mean \pm SEM	Median	Range	
Total no. of samples analyzed	304				
Can3cers	164	63.707 \pm 0.746	63.5	43-86	0.273
Normal	140	60.214 \pm 1.344	64	17-98	
Caucasian					
Cancers	84	59.750 \pm 0.888	59.5	37-83	0.736
Normal	80	57.244 \pm 1.955	61	17-98	
African American					
Cancers	80	67.862 \pm 1.028	68	43-86	0.073
Normal	60	63.952 \pm 1.6	67	20-94	

APPENDIX

Table 2: Population Diversity and genotype of normal but ethnically diverse populations from *NCBI dbSNP* database for MxA polymorphism rs2071430. Table obtained taken from (220) (245)

rs2071430 (-88G/T)				Genotype			Alleles	
<u>ss#</u>	<u>Population</u>	<u>Ethnicity</u>	<u>Chr. Sample #</u>	<u>G/G</u>	<u>G/T</u>	<u>T/T</u>	<u>G</u>	<u>T</u>
ss48295846	PI	anonymous samples	204	0.725	0.225	0.050	83.80%	16.20%
	CAUC1	Caucasian heritage	62	0.839	0.161	0.000	91.90%	8.10%
	AFR1	African/African American	48	0.708	0.250	0.042	83.30%	16.70%
	HISP1	Hispanic heritage	46	0.870	0.130	0.000	93.50%	6.50%
	PAC1	Pacific rim heritage	48	0.458	0.375	0.167	64.60%	35.40%

APPENDIX

Table 3: Association of MxA rs2071430 with prostate cancer. Genotype distribution for all samples, corresponding odds ratio, 95% confidence interval and p values are shown (OR= odds ratio, CI= confidence interval, N= number of samples and %= percent samples) Table taken from (220)

Geno type	All Samples		Cancer		Normal		OR	95% CI Lower	95% CI Upper
	N	%	N	%	N	%			
GG	200	65.8	118	72	82	58.6	1.713	1.041	2.818
GT	92	30.3	42	25.6	50	35.7	Reference		
TT	12	3.9	4	2.4	8	5.7	0.60	0.167	2.116
Total	304	100.0	164	100.0	140	100.0			

APPENDIX

Table 4: Association of MxA rs2071430 with prostate cancer among race. Genotype distribution for Caucasian samples and African-American samples and corresponding odds ratio and 95% confidence interval are shown (OR= odds ratio, CI= confidence interval) Table obtained from (220)

Genotype	Race	Cancer	%	Normal	%	OR	95% CI Lower	95% CI Upper
GG (n=200)	African American	52	26	30	15	1.37	0.769	2.442
	Caucasian	66	33	52	26	Reference		
GT (n=92)	African American	26	28.2	22	24	2.07	0.897	4.780
	Caucasian	16	17.4	28	30.4	Reference		
TT (n=12)	African American	2	16.6	8	66.6	0.0625	0.0019	1.998
	Caucasian	2	16.6	0	0	Reference		

APPENDIX

Figure 15: Meta-analysis of MxA Expression in Metastatic Prostate cancer. GEO

Profiles ID: 34859781 Expression profiling by array, count, 171 samples. Expression of MxA is decreased in metastatic prostate cancer [red rectangle] as compared to normal tissue or normal tissue adjacent to tumor [blue rectangle]. Figure adopted from (246)

APPENDIX

Figure 16

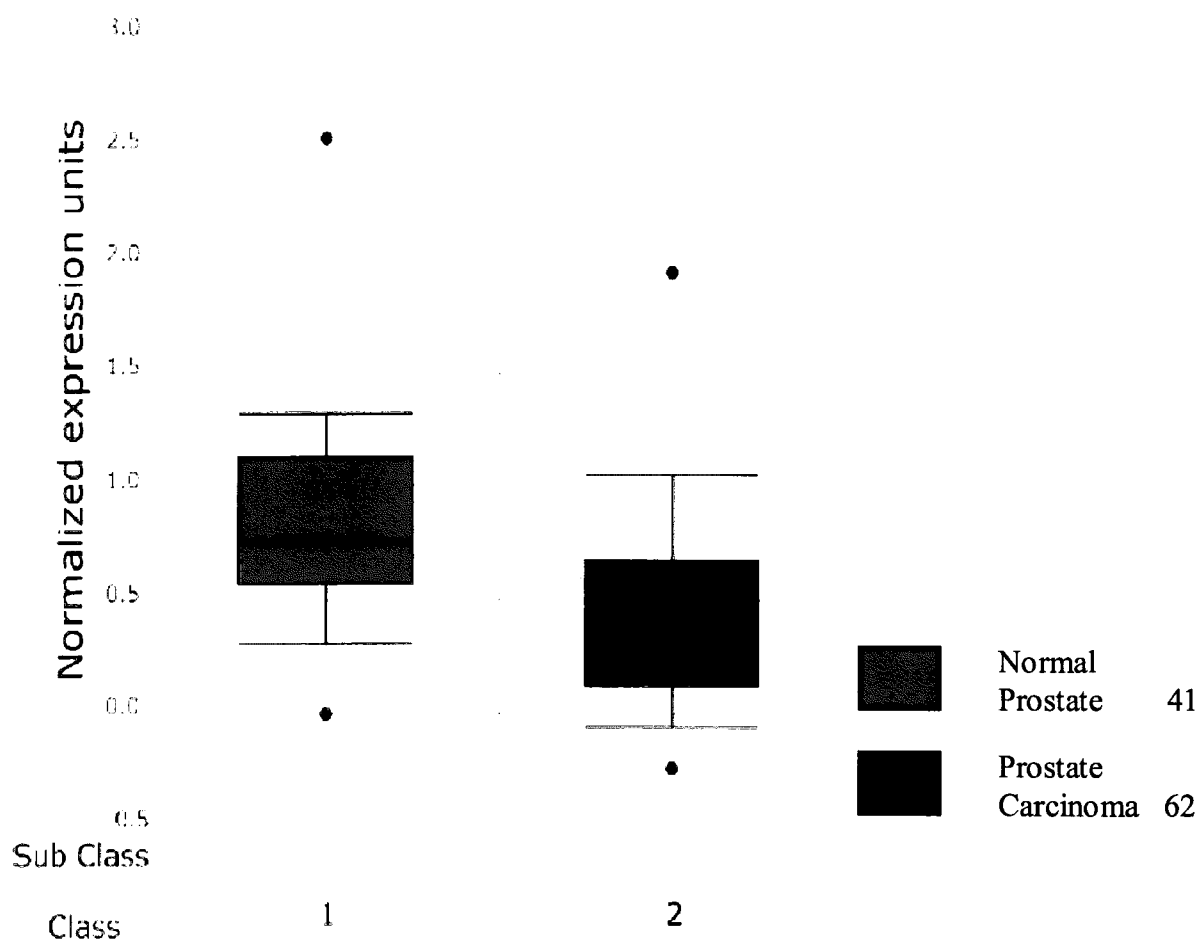
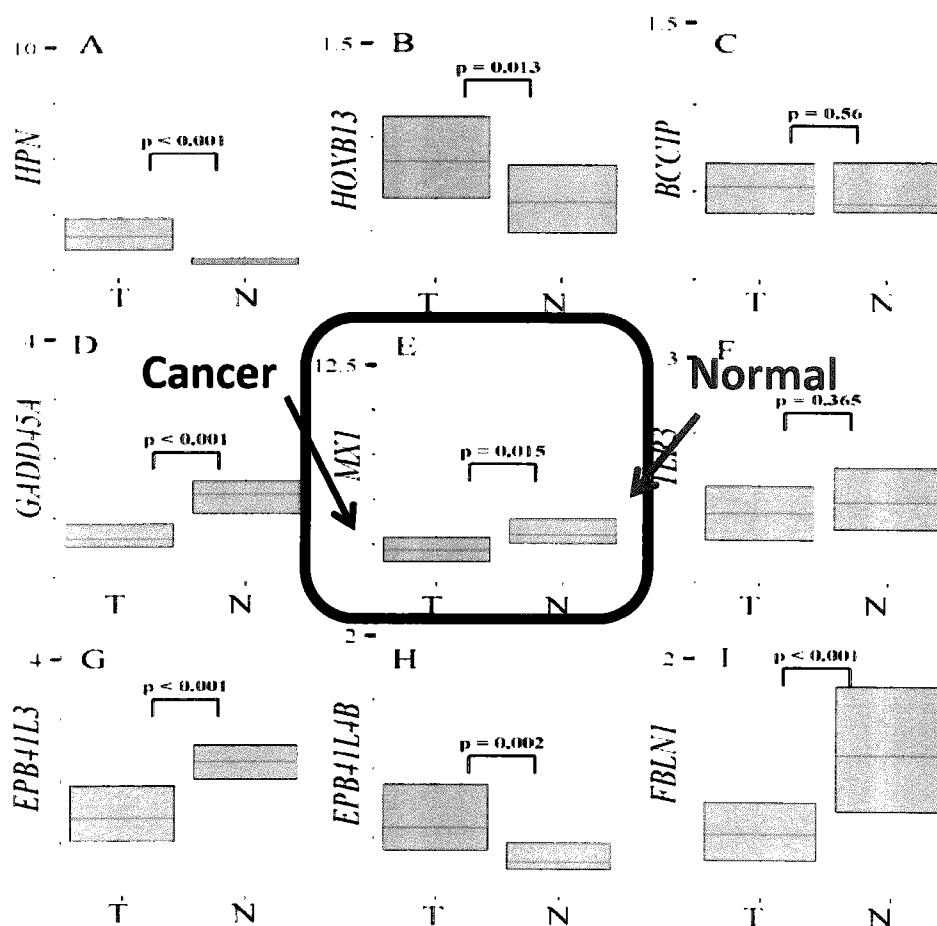


FIGURE 16: Oncomine database meta-analysis illustrates decreased expression of MxA in prostate carcinoma. Expression of MxA is high in normal prostate tissues. Figure taken from (247)

APPENDIX

Figure 17



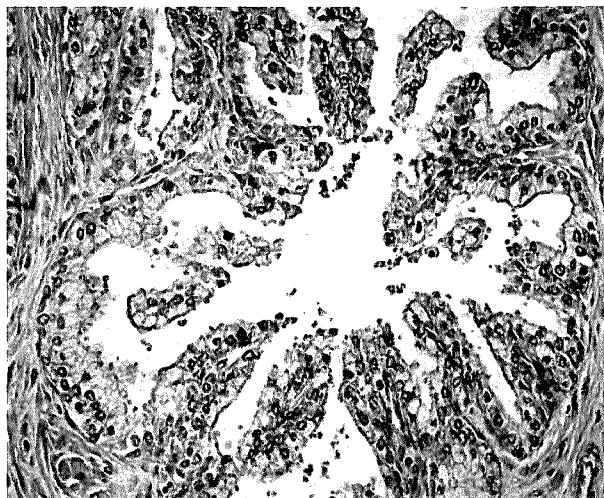
APPENDIX

FIGURE 17: Box plot comparison of expression of selected genes in prostate cancer vs. benign tissues by real-time quantitative RT-PCR. A: HPN; B: HOXB13; C: BCCIP; D: GADD45A; E: MxA; F: TLR3; G: EPB41L3; H: EPB41L4B; I: FBLN1. Expression values were determined for each sample in duplicate with < 10% variation. They are indicated relative to the reference gene TBP determined in the same fashion. T: cancer samples (n = 47), N: benign tissue samples (n = 13); p-values according to Mann-Whitney tests. Figure used from (219). MxA is significantly decreased in prostate cancer tumors as compared to benign tissue samples.

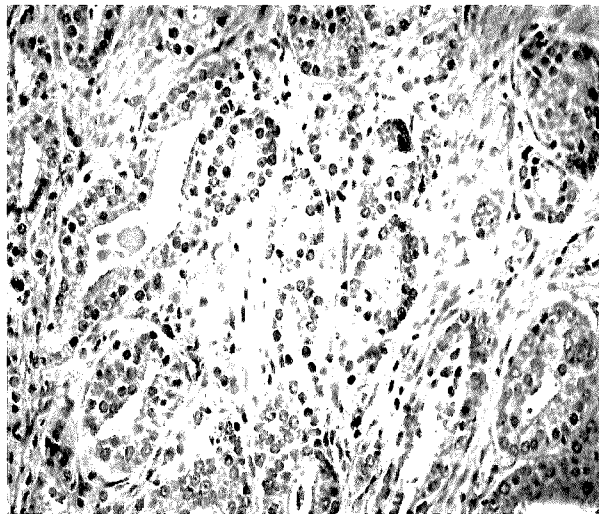
APPENDIX

Figure 18

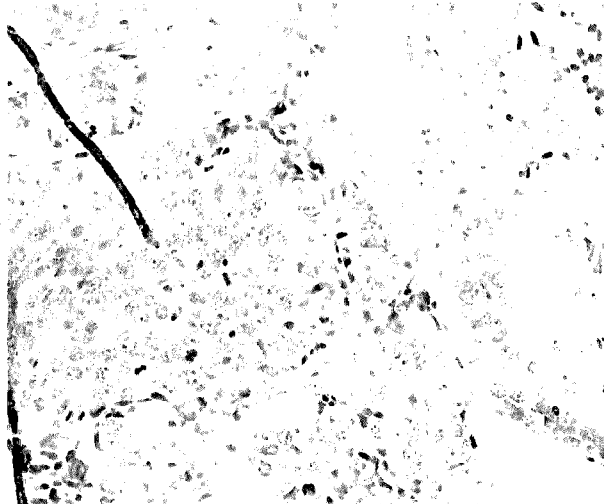
Normal Middle Lobe



Adenocarcinoma Grade 1



Adenocarcinoma Grade 2



APPENDIX

FIGURE 18: Immunohistochemistry (20X) of MxA expression in a BioMax prostate tissue microarray. Expression of MxA high in normal tissue; however, as the grade of adenocarcinoma increases the expression of MxA decreases.

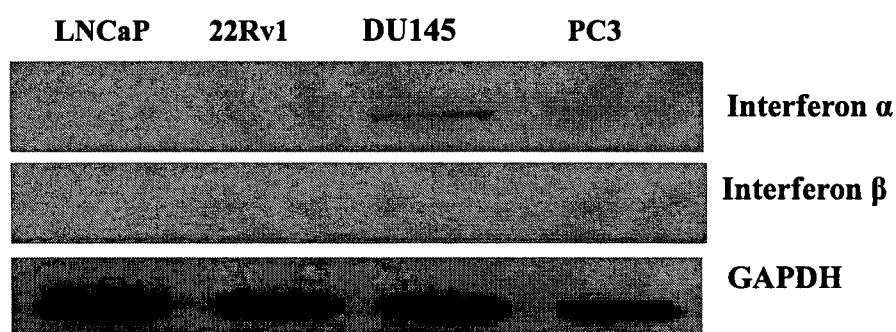
APPENDIX**Figure 19**

FIGURE 19: RT-PCR of Interferon levels in prostate cancer cells. All prostate cancer cells do not produce their own endogenous interferons with the exception of DU145 and PC3. These cells produce endogenous IFN α only.

APPENDIX

Figure 20

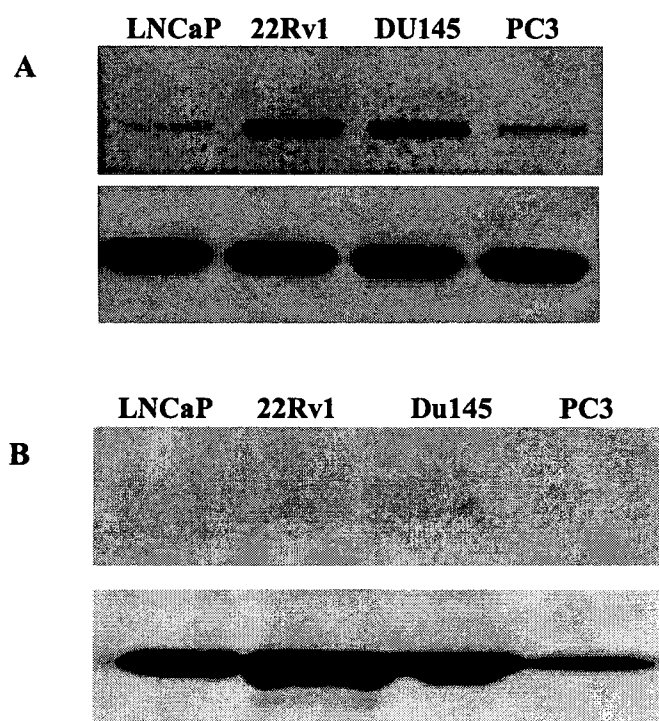


FIGURE 20: Expression of MxA in our well established prostate cancer cell lines by A) PCR and B) Western blot analysis. There is variable expression of MxA in the cell lines as seen at the transcript level. There is expression at the protein level although overall it is relatively low.

APPENDIX

Figure 21

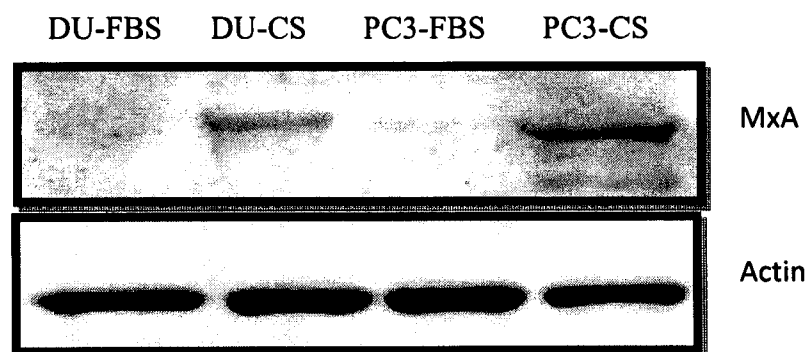
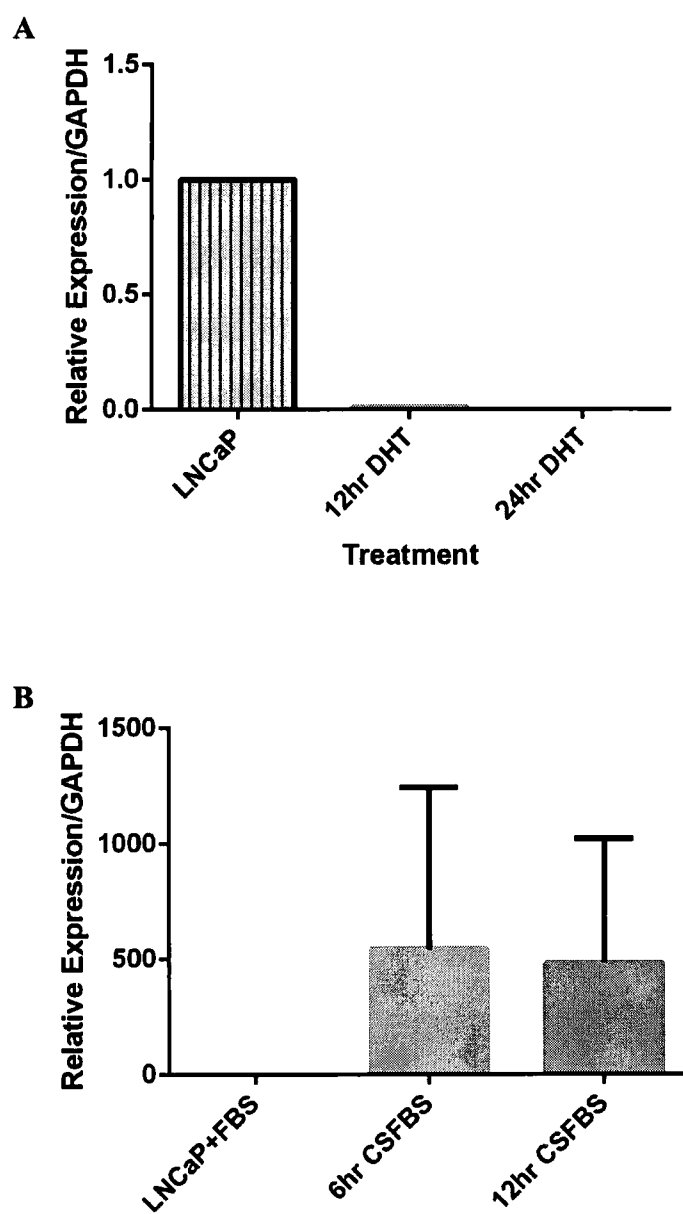


FIGURE 21: MxA is Serum Regulated. Western blot analysis illustrates MxA expression in prostate cancer cell lines cultured in different serum. Cells cultured in fetal bovine serum (FBS) resulted in decreased expression of MxA. Calf serum (CS) produces constitutive expression of MxA protein.

APPENDIX

Figure 22

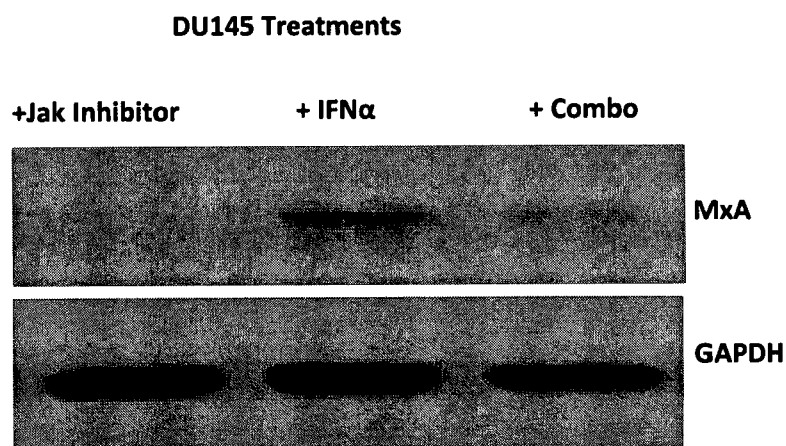


APPENDIX

Figure 22: Regulation of MxA by Androgens. A) qPCR analysis of MxA expression after 12hr and 24hr DHT treatment respectively. Androgens down-regulate the basal expression level of MxA. B) qPCR illustrates culturing the cells in serum devoid of androgens results in an increase in MxA expression.

APPENDIX

Figure 23



Effects of 300U of IFN α and 15nM Jak Inhibitor on MxA expression in DU145 cells

Figure 23: Interferon Regulation of MxA. RT-PCR illustrates cells with constitutive MxA expression (DU145) are sensitive to the classical interferon signaling pathway. Treating the cells with an inhibitor of this pathway results in loss of expression but treating with exogenous IFN alpha re-expresses MxA.

APPENDIX

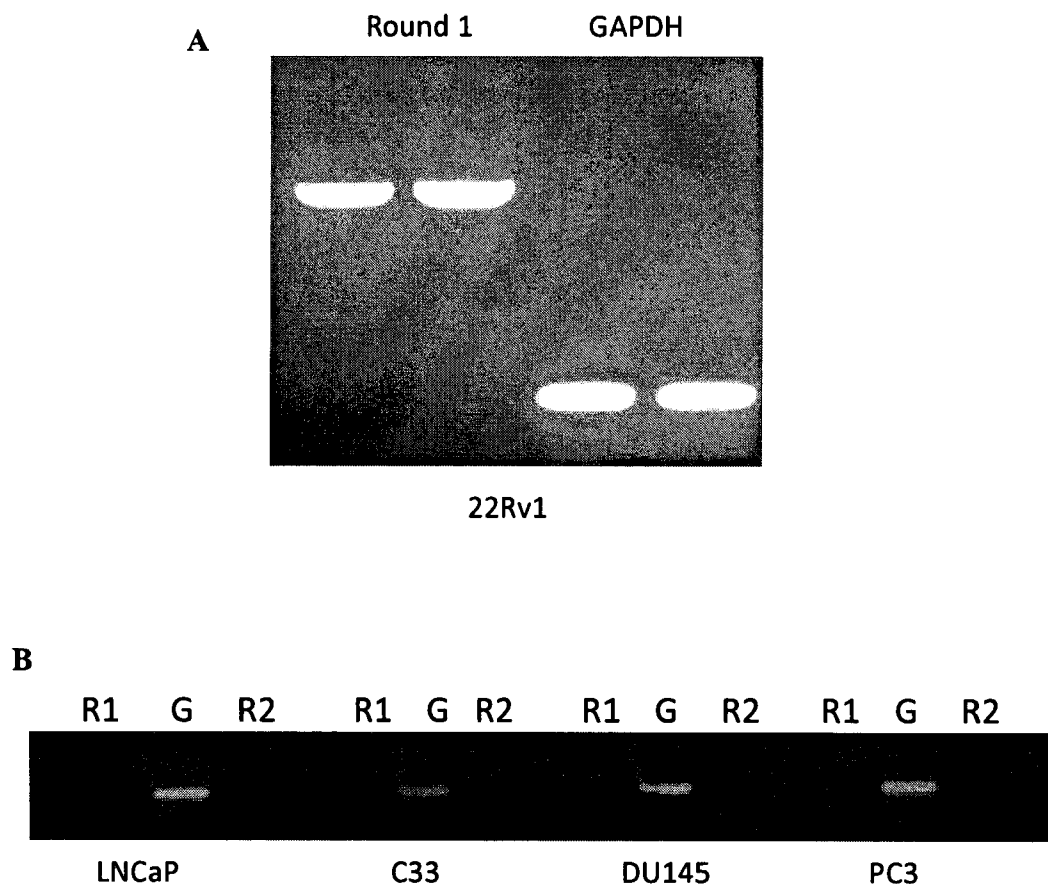
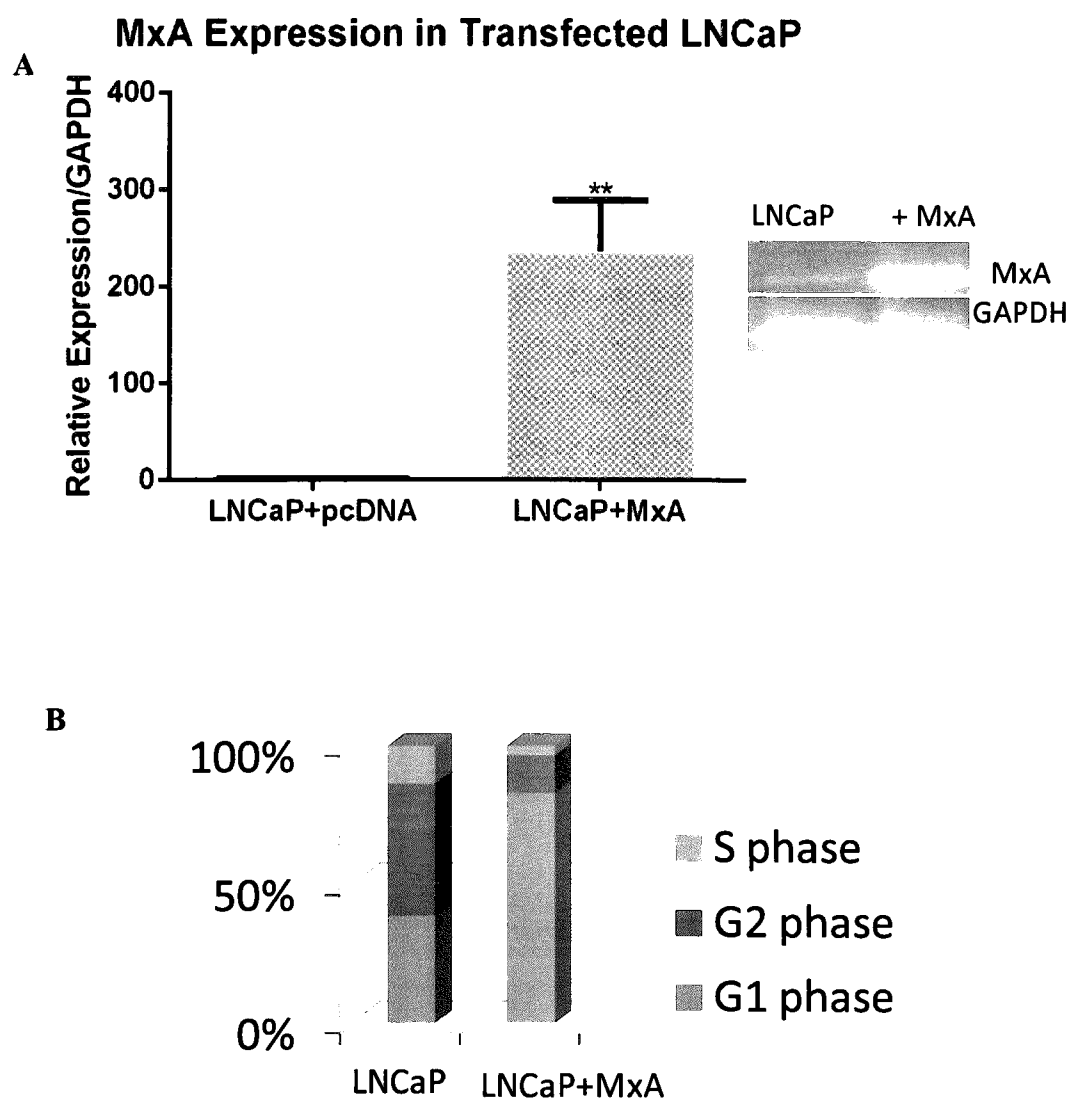
Figure 24

Figure 24: Viral regulation of MxA. RT-PCR of prostate cancer cell lines for detection of XMRV virus production. All cells were negative for XMRV virus with the exception of 22Rv1 cells which are known to produce high titer of the virus. 22Rv1 also have high expression of MxA and do not produce their own interferons; thereby, illustrating viral dependent regulation of MxA. R1: First round of nested PCR, G: GAPDH, R2: Second round of nested PCR.

APPENDIX

Figure 25

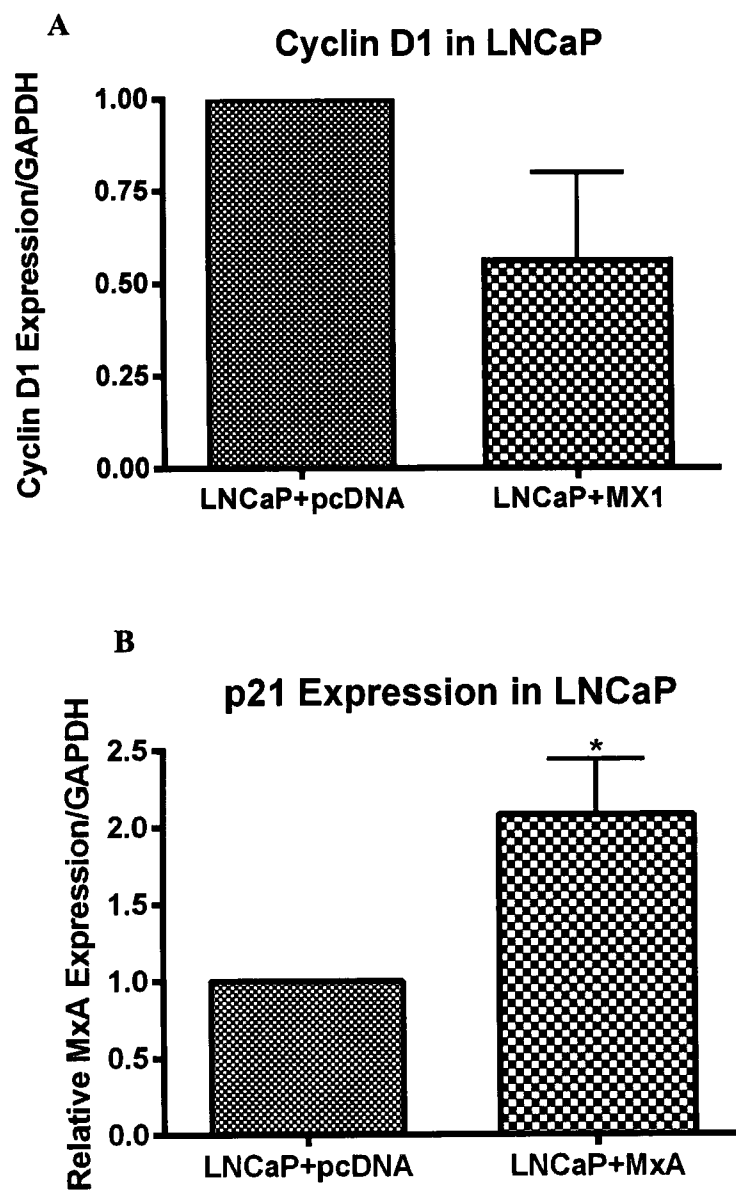


APPENDIX

Figure 25: Over-expression of MxA in LNCaP cells. A) qRT-PCR analysis shows successful transient over-expression of MxA in LNCaP cell. B) The over-expression of MxA in LNCaP cells had a profound effect on the cell cycle. There is a G₁ cell cycle arrest when MxA is overexpressed.

APPENDIX

Figure 26



APPENDIX

Figure 26: Effects of Gain of MxA expression on cell cycle. A) qRT-PCR illustrates a decrease in Cyclin D1 expression in cells constitutively expressing MxA. B) p21 was up-regulated in LNCaP+MxA cells as compared to vector controls. The down-regulation of Cyclin D1 by an increase in p21 results in the cell cycle arresting at G₁.

APPENDIX

Figure 27

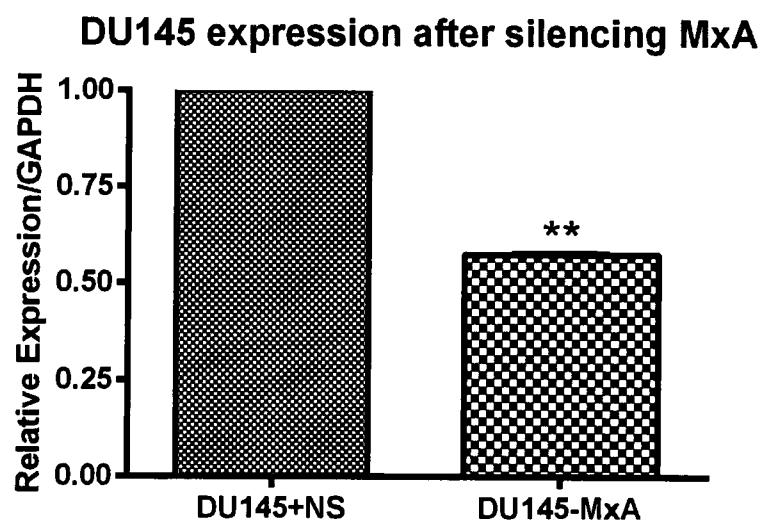


Figure 27: Stable knockdown of MxA in DU145 prostate cancer cells. DU145 cells were stably transfected and subjected to antibiotic selection. Cells were subjected to qRT-PCR to determine expression of MxA. MxA expression was knocked-down.

APPENDIX

Figure 28

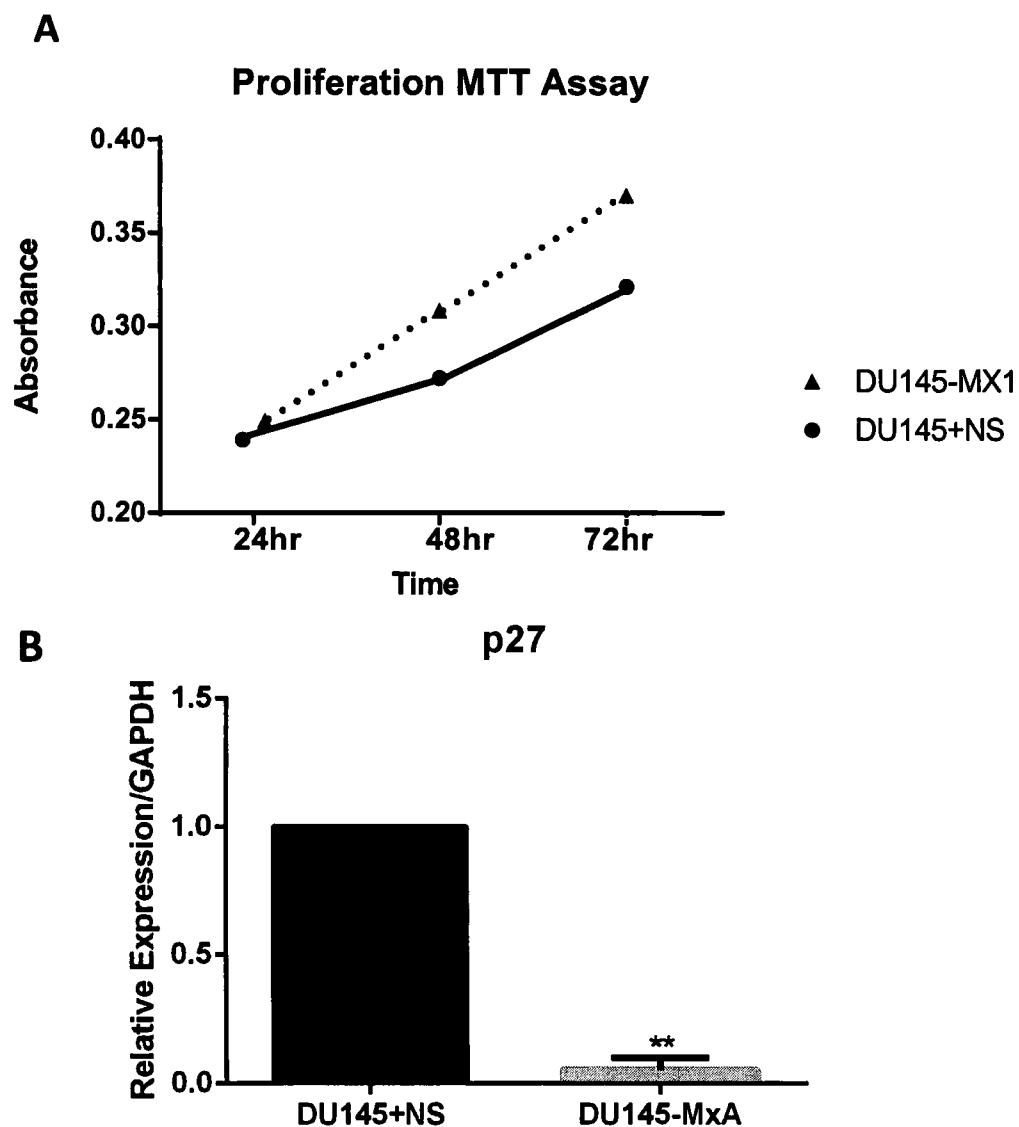
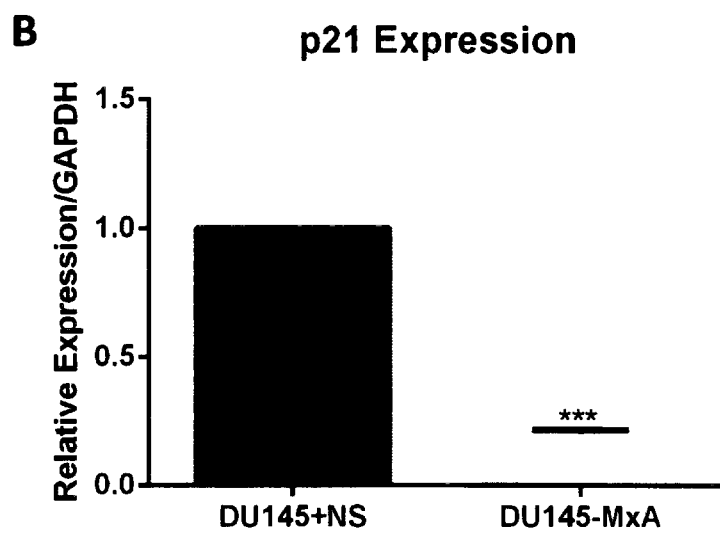
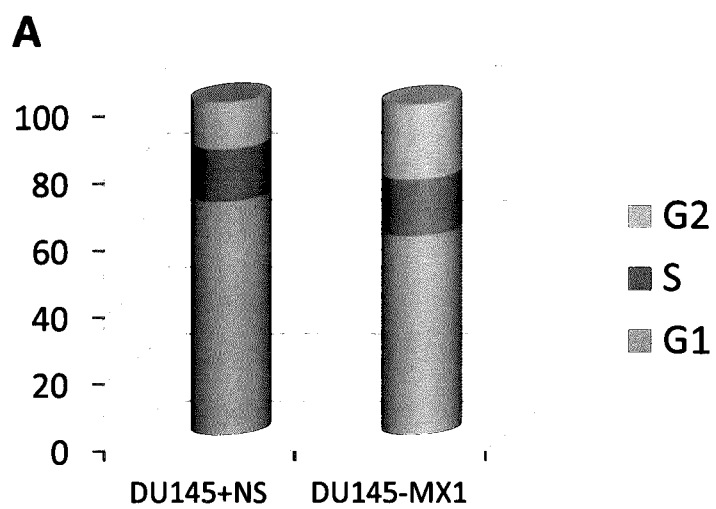


Figure 28: Effect of Loss of MxA on Proliferation. A) MTT proliferation assay illustrates a slight increase in proliferation when MxA is stably knocked down. B) Expression of p27 (anti-proliferative) is decreased when MxA is decreased

APPENDIX

Figure 29

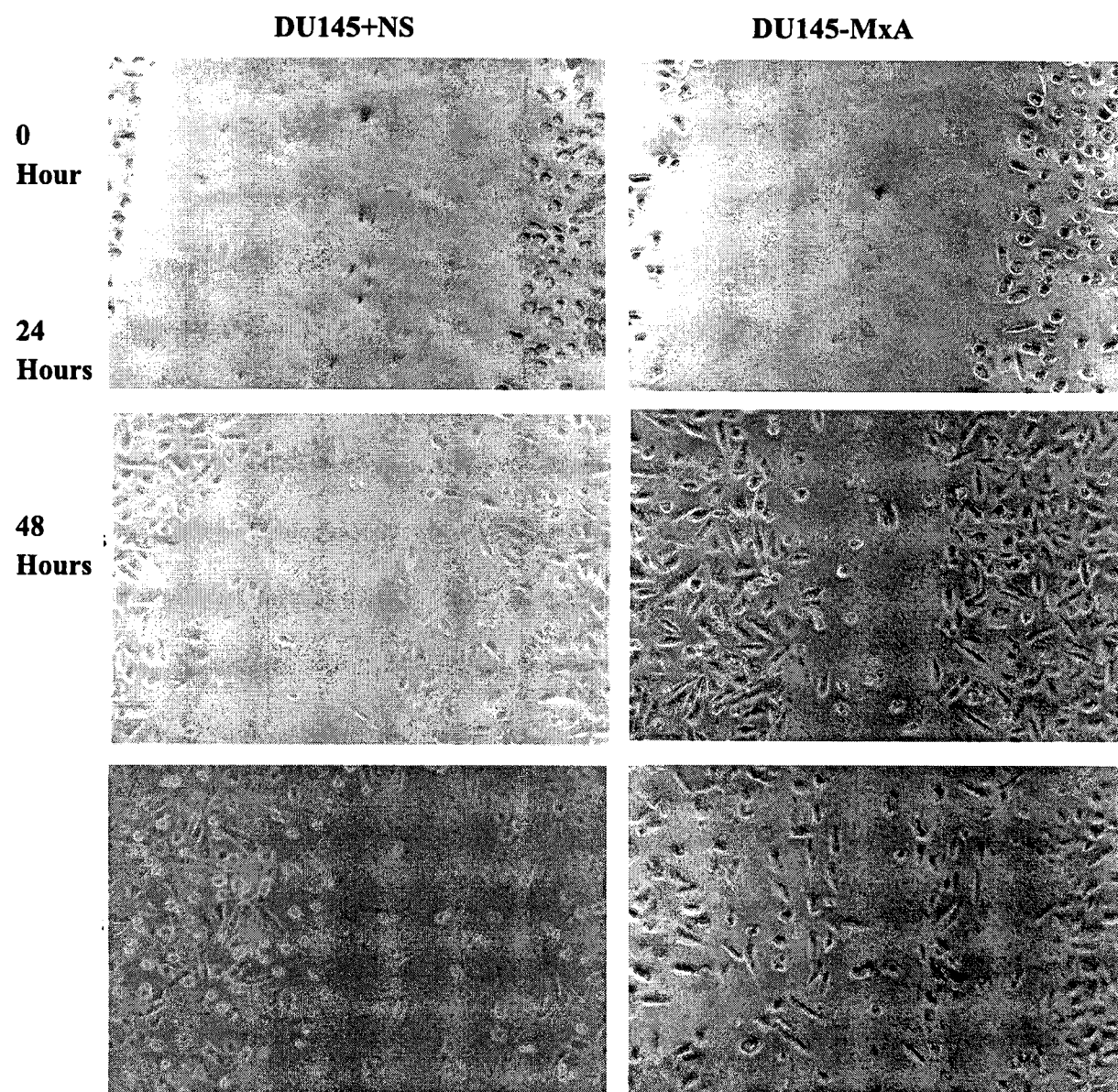


APPENDIX

Figure 29: Cell Cycle Analysis of DU145-MxA. A) Flow cytometry revealed an increase in G₂ of the cell cycle in cells with decreased MxA as compared to the Non-Specific vector control. B) Expression of the CDKI p21 is decreased in DU145-MxA cells. This data supports the observation that decreased MxA results in increased proliferation.

APPENDIX

Figure 30



APPENDIX

Figure 30: Scratch Wound Assay. DU145+NS and DU145-MxA cells were plated in 6-well cell culture plates and a wound was created using a 20 μ L pipette tip. The cells were allowed to heal over a period of 48 hours with pictures of random fields taken at 0, 24, and 48 hours respectively. Cells with decreased MxA were able to heal the wound at a faster rate as compared to the vector control.

APPENDIX

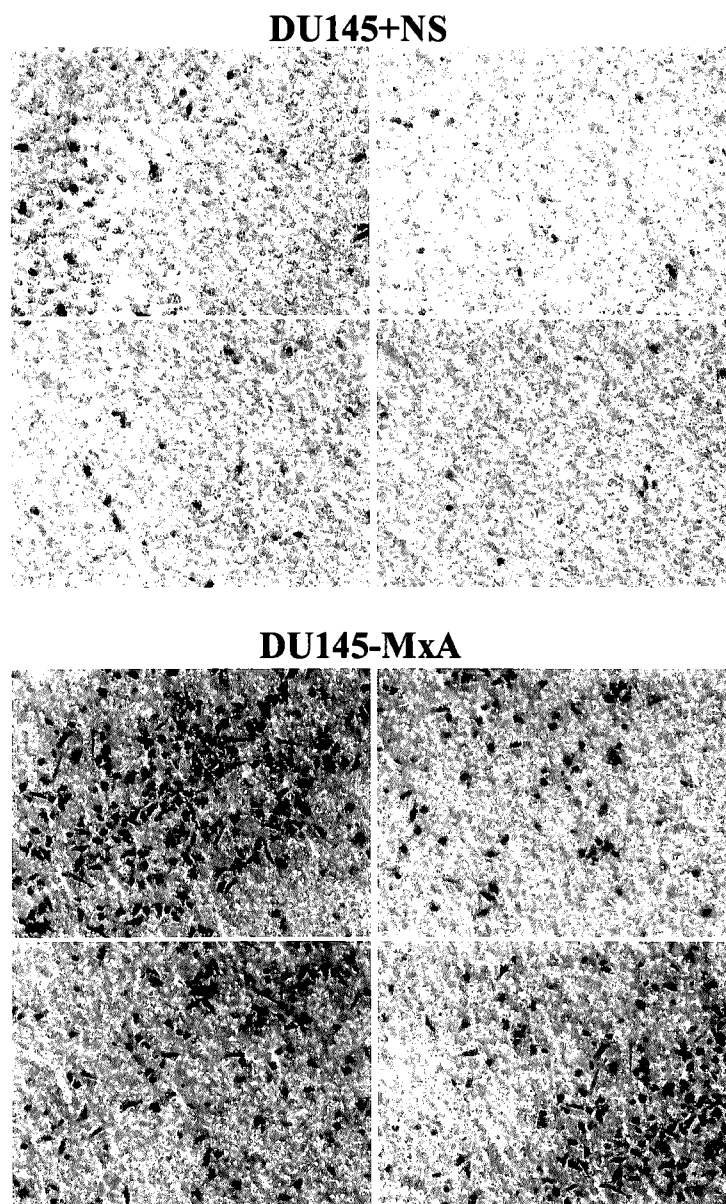
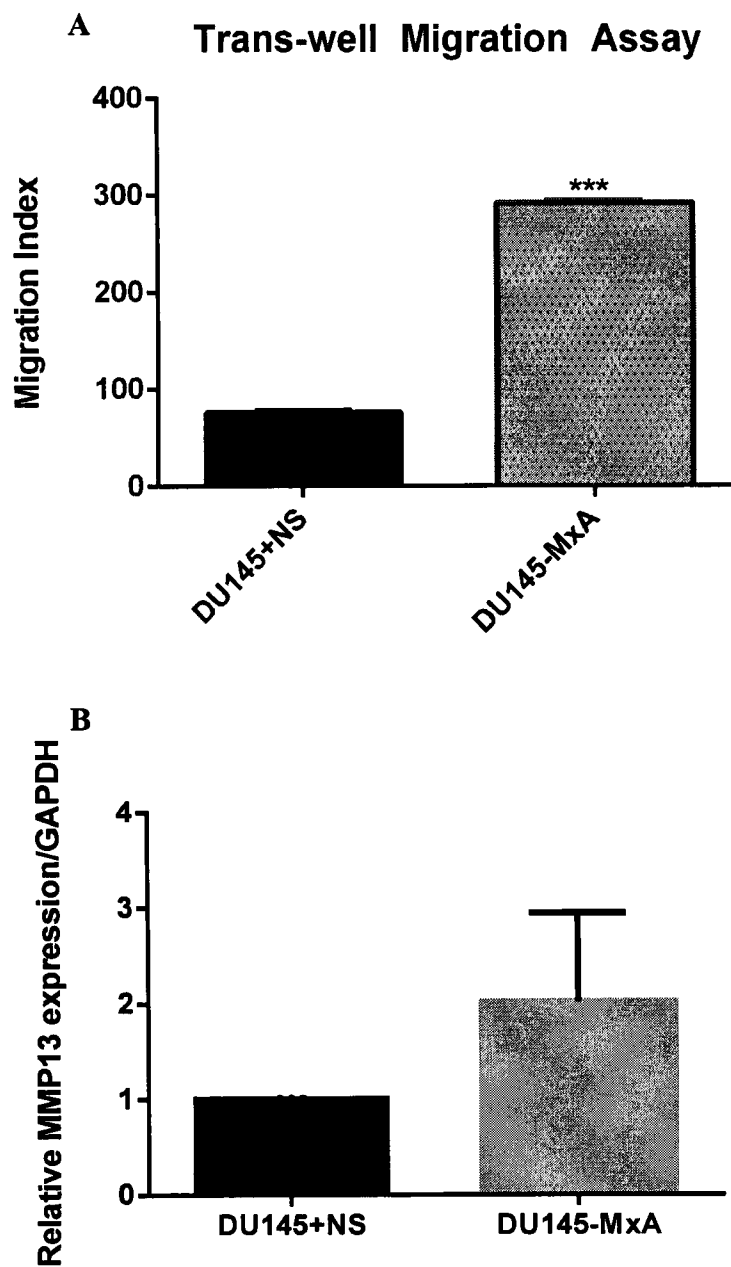
Figure 31

Figure 31: Trans-well migration images of DU145 transfected cells. Migratory cells are stained purple.

APPENDIX

Figure 32



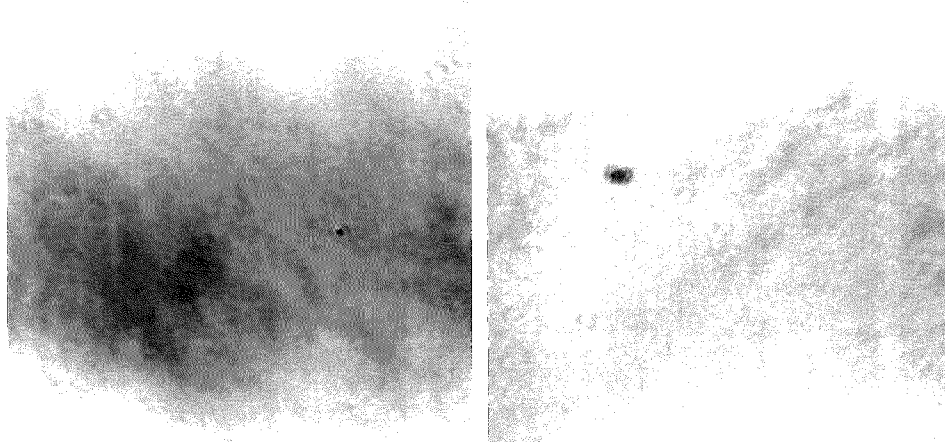
APPENDIX

Figure 32: Trans-well migration assay and MMP analysis. A) Quantitation of migration index. DU145-MxA exhibited an increased migratory index as compared to cell with constitutive MxA expression. B) Cells expressing constitutive MxA (DU145+NS) have decreased levels of MMP13 which is consistent with reduced migration/motility.

APPENDIX

Figure 33**A**

DU145+NS

**B**

DU145-MxA

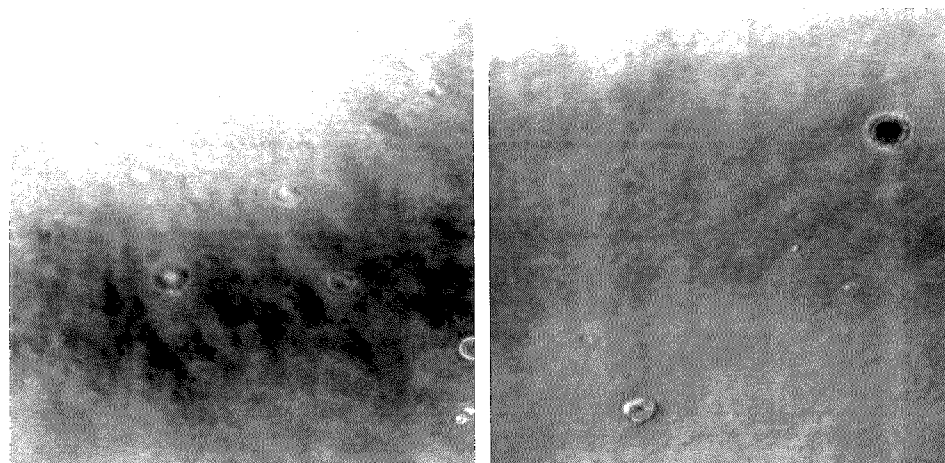


Figure 33: Soft Agar Assay Images. Microscopic images of DU145+NS and DU145-MxA cells after 3 weeks of anchorage independent cell growth.

APPENDIX

Figure 34

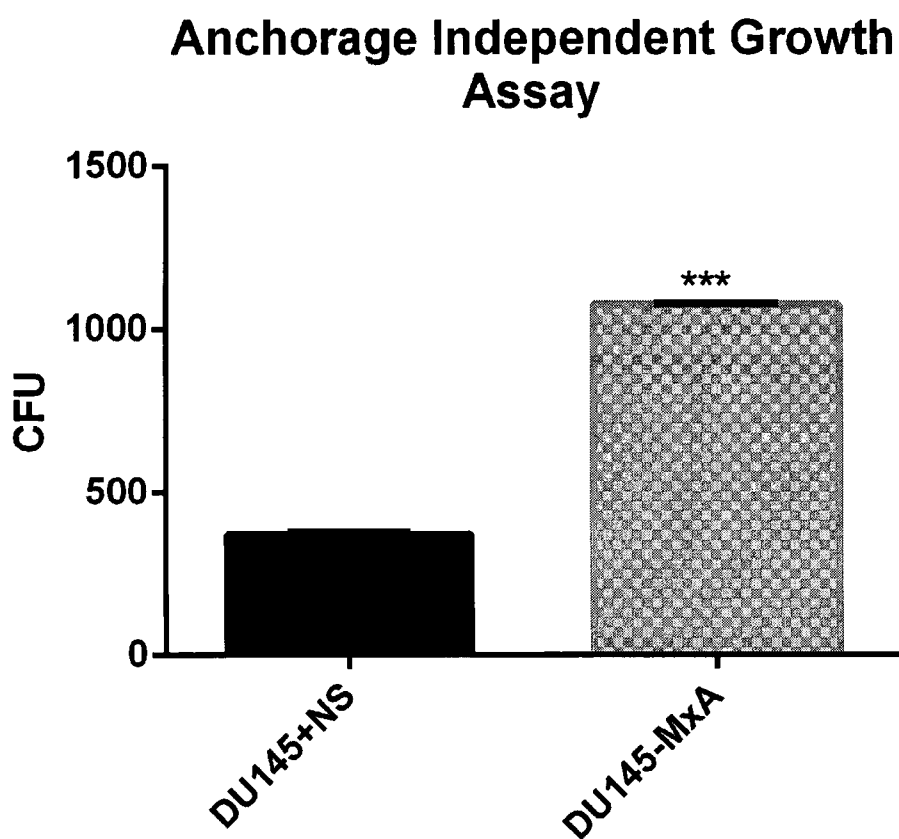


Figure 34: Quantitation of Anchorage Independent Cell Growth. DU145-MxA cells had greater colony forming units as compared to the vector control cell. These results illustrate MxA's anti-tumorigenic potential.

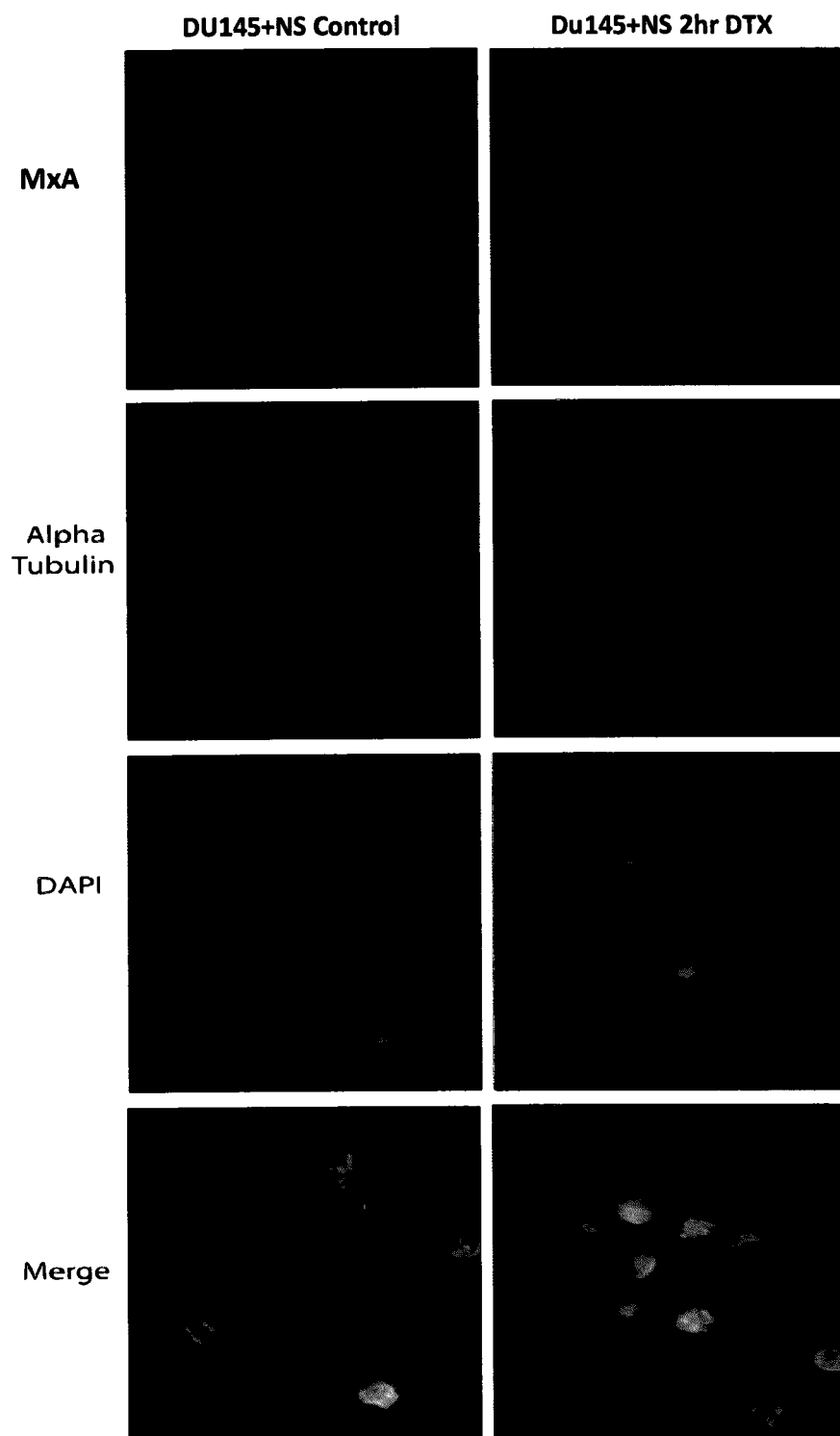
APPENDIX**Figure 35**

Cell lines (post DTX)	% Live	% Apoptotic	% Dead
DU145+NS	58.5	37.5	3.7
DU145-MxA	76.6	18.8	4.2

Figure 35: Annexin V Apoptosis Assay. DU145 transfected cells were treated with 1 μ M DTX for 24 hours. Annexin V staining was done and the results were analyzed using FACS analysis. Cells with constitutive MxA have increased apoptotic cells as compared to silenced MxA cells. The reduction of apoptosis in DU145-MxA cells provides evidence that MxA sensitizes prostate cancer cells to the chemotherapeutic drug Docetaxel (DTX)

APPENDIX

Figure 36



APPENDIX

Figure 36: Immunocytochemistry of DTX treatment in DU145 cells that express MxA.

Left panel: No DTX treatment. Right panel: 2hr DTX treatment. MxA did co-localize with alpha-Tubulin before treatment with DTX and after treatment that localization was disrupted. MxA had very diffuse cytoplasmic staining before treatment (left); however, after treatment MxA was localized in punctate dots (right). This intriguing observation will be investigated further but may provide some mechanism of how MxA sensitizes these cells to chemotherapeutic agents.

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